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(54) Title: NEWLY IDENTIFIED CHOLINEPHOSPHOTRANSFERASES AND ETHANOLAMINEPHOSPHOTRANS-  
FERASES

(57) Abstract: The present invention relates to polypeptides comprising one or more of the amino acid motifs selected from the group consisting of a sequence with at least 80% identity to any of (a) P-L-X-D-X(35,75)-R-R-X(8)-[YF]-X(2)-R-X(6)-T (b) C-X-D-X(3)-S-G-H-T (c) H-Y-[TS]-X-D-[VI]-X(3)-[FYI]-X(6)-F-X(2)-Y-H, which transfer phosphocholine and phosphoethanolamine, nucleotide sequences coding for any of these polypeptides and nucleotide sequences complementary thereto, plasmids, vectors and a (micro)organism or cell comprising said nucleotide sequences. Furthermore, the present invention relates to processes to produce cholinephosphotransferases and ethanolaminephosphotransferases like sphingomyelin synthase, ethanolamine phosphorylce-ramide synthase, phosphatidylcholine:glycoprotein cholinephosphotransferase and phosphatidylcholine:glycolipid cholinephosphotransferase. The present invention also provides the application of said nucleotide sequences to influence the equilibrium reactions or to develop compounds influencing the equilibrium reactions wherein said transferases are involved and the application of said compounds in medical use. Finally, a process has been provided to isolate candidates for functional genes of a previously unidentified enzyme from a large database by isolating candidates for functional genes of a previously unidentified

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transduction and membrane trafficking (Simons and Toomre, 2000; Holthuis et al., 2001). Since the discovery of the 'SM cycle' as a putative signaling system analogous to well-known second messenger systems like the phosphoinositide pathway, SM has emerged to the focus of interest in many research laboratories (Kolesnick and Hannun, 1999; Andrieu-Abadie and Levade, 2002).

Since the enzyme SM synthase is also able to catalyse the reverse reaction, namely the formation of PC from SM and DAG (Marggraf and Kanfer, 1984, van Helvoort et al., 1994) it may regulate, in opposite directions, the cellular levels of the bioactive lipids ceramide and DAG. The latter two are important regulators of membrane trafficking, cell proliferation and apoptosis (Kolesnic and Hannun, 1999; Bankaitis, 2002; Brose and Rosenmund, 2002, Pettus et al., 2002). Hence, one may anticipate that the physiological significance of SM synthase goes beyond the formation of SM.

The subcellular localisation of the enzyme has been the subject of numerous studies. After the initial debate had focused on whether SM synthase is located in the Golgi or at the plasma membrane (Marggraf et al.; Voelker and Kennedy, 1982; Lipsky and Pagano, 1985), subsequent work revealed evidence for the presence of SM synthase activity in both membranes (Futerman et al., 1990; Jeckel et al., 1990; van Helvoort et al., 1994). Whether SM synthesis detected at these locations is due to the presence of more than one isoform of the enzyme has remained an open issue.

Progress in understanding the biological roles of SM synthesis and its regulation is hampered by the fact that no successful purification of the responsible enzyme has been achieved. Recent work has identified a bacterial SM synthase released from *Pseudomonas aeruginosa* (Luberto et al., 2003). However, this activity is a soluble protein, hence in contrast to the animal enzyme that is tightly associated with the membrane (Ullman and Radin, 1974; Voelker and Kennedy, 1982). Other efforts focused on the isolation of SM synthase mutants by screening Chinese hamster ovary cells for resistance to a SM-

10 Definitions

- similarity: percentage of identical amino acids or amino acids with similar properties between two sequences.

30 *Schizosaccharomyces pombe* (Sp)



- Candida albicans* (Ca)
- sphingomyelin (SM) N-Acyl-D-sphingosine-1-phosphocholine
- sphingomyelinase (SMase)
- phosphatidyl choline (PC) 1,2-Diacyl-sn-glycero-3-phosphocholine
- 5 diacyl glycerol (DAG) sn-1,2-Diacyl glycerol
- ceramide (CER) N-Acyl-D-sphingosine
- inositol phosphorylceramide (IPC)
- lipid phosphate phosphatase (LPP)
- N-(7-(4-nitrobenzo-2-oxa-1,3-diazole)aminocaproyl)sphingosine (NBD-CER)
- 10 N-ethylmaleimide (NEM)
- electrospray ionization tandem mass spectrometry (ESI-MS/MS)
- phytoceramide-based SMs (phytoSM)
- phosphorylcholine (Ch-P)
- cytidine-5' diphosphocholine-choline (CDP-Ch)
- 15 tubovesicular membranes (TVM)
- phosphatidylethanolamine (PE)
- phosphatidylinositol (PI)
- phosphatidylserine (PS)
- phosphatidic acid (PA)
- 20 phosphatidylglycerol (PG)
- open reading frame (ORF)
- CSS1, CSS2 and CSS3 (for candidate SM synthase family 1-3),
- with human CSS3 $\alpha$ 1 and CSS3 $\alpha$ 2 renamed as *huSMS1* and
- 25 *huSMS2* and *C. elegans* CSS3 $\alpha$ 1.1 and *C. elegans* CSS3 $\alpha$ 2 renamed
- as *ceSMS1* and *ceSMS2*

-- with human CSS3 $\beta$  renamed as *huSMSr* (SMS related), with mice CSS3 $\beta$  renamed as *moSMS1*, with *C. elegans* CSS3 $\beta$  as *ceSMS1* and *Drosophila* CSS3 $\beta$ , as *doSMS1*  
 -- with *C. elegans* CSS3 $\gamma$  renamed as *ceSMSdr*, (SMS distantly related).

#### Description of the figures with legends.

**Fig. 1. Selection and phylogenetic analysis of candidate SM synthases.**

10 (A)

Animal entries in SwissProt/TrEMBL were searched for the presence of a sequence motif shared by LPPs and Aurlp proteins and then further selected on the basis of three additional criteria, as indicated. (B) Phylogenetic tree of human candidate SM synthases (CSS) and previously characterised members of the human LPP super family. (C) Phylogenetic tree of CSS proteins from human (*Hu*), mouse (*Mm*), *Caenorhabditis elegans* (*Ce*) and *Drosophila melanogaster* (*Dm*), and of Aurlp proteins from *Saccharomyces cerevisiae* (*Sc*), *Schizosaccharomyces pombe* (*Sp*) and *Candida albicans* (*Ca*). Asterisks denote CSS proteins expressed in *S. cerevisiae* and tested for SM synthase activity.

20 SM synthases (SMS) are marked in red. SwissProt/TrEMBL accession numbers of CSS proteins are: (1) Q9TYV2; (2) Q20696; (3) Q9VS60/Q9VS61; (4) Q96LT4; (5) Q9DA37; (6) Q86VZ5; (7) Q8VCQ6; (8) Q8NHU3; (9) Q9D4B1; (10) Q9XTV2; (11) Q20735; (12) Q965Q4; (13) Q9D606; (14) Q9NXE2; (15) Q8VCY8; (16) Q96GM1; (17) Q96MP0; (18) AAP57768; (19) Q9BQF9; (20) AAP57767; (21) Q22250; (22) Q9TXU1; (23) Q9VNT9; (24) Q9VNU1; (25) Q10022; (26) Q9D4F2; (27) Q8IY26; (28) Q91WB2; (29) Q96SS7; (30) Q8T8T9; (31) Q22461. Note that (4) Q96LT4 contains a partial protein sequence and that the complete ORF was deduced from a corresponding EST clone (see the Table).

30

**Fig. 2.** A subset of CSS3 family members displays SM synthase activity upon expression in yeast. **(A)** Immunoblots of cells expressing various CSS proteins were stained with antibodies recognising the V5 epitope-tagged carboxy termini of CSS proteins. Control denotes cells transformed with empty vector. **(B)** TLC separation of reaction products generated when NBD-ceramide (NBD-Cer) was incubated with lysates of control or CSS-expressing cells in the presence (+) or absence (-) of IPC synthase inhibitor, aureobasidin A (Aba). **(C)** Metabolic labelling of cells expressing human CSS3 $\alpha$ 1/SMS1 or CSS3 $\alpha$ 2/SMS2 with [ $^{14}$ C]-choline and NBD-ceramide. The lipids were extracted, separated by two-dimensional TLC and analysed for fluorescence and radioactivity. Note that SMS1- and SMS2-expressing cells, but not control cells, synthesized NBD-SM, and that this NBD-SM was labelled with [ $^{14}$ C]-choline. **(D)** Metabolic labelling of cells expressing human CSS3 $\alpha$ 2/SMS2 with [ $^{14}$ C]-choline. The lipids were extracted, deacylated by mild alkaline hydrolysis (+NaOH) or control incubated (-NaOH) and separated by two-dimensional TLC before autoradiography. Note that SMS2-expressing cells synthesized alkaline-resistant species of [ $^{14}$ C]-choline-labelled lipids (phytoSM) that were absent in control cells.

**Fig. 3.** NEM inhibits yeast-associated SMase but not mammalian SM synthase. **(A)** TLC separation of reaction products generated when NBD-SM (1.8  $\mu$ M) was incubated with lysates of exponentially grown yeast cells (20 OD<sub>600</sub> cells) in the presence (+) or absence (-) of *N*-ethylmaleimide (NEM; 1 mM). Reactions were in 0.35 ml RB1 buffer containing 1  $\mu$ g/ml of IPC synthase inhibitor (aureobasidin A) and incubated for 30 min at 37 °C. **(B)** TLC separation of reaction products generated when NBD-ceramide (NBD-Cer; 1,8 mM) was incubated with lysates of HeLa cells (1/1: 2 x 10<sup>6</sup> cells; 1/4: 0.5 x 10<sup>6</sup> cells) in the presence (+) or absence (-) of NEM (1 mM). Reactions were in 0.35 ml PNS buffer, and incubated for 30 min at 37 °C. Lipid extraction and TLC analysis were as described in Materials and methods.

**Fig. 4.** Detergent extracts of human CSS3 $\alpha$ 2/SMS2-expressing yeast cells support SM formation from bovine brain ceramides. Lines **A** and **B** show the reconstituted ion chromatograms of  $m/z$  731.6, corresponding to the  $m/z$  ratio of protonated 18:0 SM, during the separation of molecular species of choline-containing phospholipids after solid phase extraction of membrane extracts that had been incubated with bovine brain ceramides and egg PC. The elution time of an authentic standard of 18:0 SM is indicated in the chromatogram by an arrow (42.3 minutes). Line **A** was derived from detergent extracts of human CSS3 $\alpha$ 2/SMS2-expressing cells and line **B** from detergent extracts of control cells. Mass spectra recorded at the elution time of 18:0 SM confirmed that formation of 18:0 SM occurred in CSS3 $\alpha$ 2/SMS2-containing extracts (top right panel), but not in control extracts (bottom right panel).

**Fig. 5.** SM synthase activity in detergent extracts of human SMS1- or SMS2-expressing yeast cells is stimulated by externally added PC. Detergent extracts of yeast cells expressing human SMS1 or SMS2 were diluted 2- to 8-fold in extraction buffer, and then incubated with NBD-ceramide (1.8  $\mu$ M) in the presence (+) or absence (-) of externally added PC (22  $\mu$ M), as described in Materials and methods. Formation of NBD-SM was monitored by one-dimensional TLC.

**Fig. 6.** Human SMS1 and SMS2 function as PC:ceramide choline phosphotransferases. (A) Detergent extracts of yeast cells expressing human SMS1, SMS2 or transformed with empty vector (control) were incubated with NBD-ceramide (1.8  $\mu$ M) in the presence or absence of different potential head group donors (22  $\mu$ M), as indicated. Formation of NBD-SM or NBD-IPC was monitored by one-dimensional TLC and quantified as described in Materials and methods. Note that addition of PI stimulated formation of NBD-IPC in all three extracts (asterisks). PC, phosphatidylcholine; SM, sphingomyelin; Ch,

choline; Ch-P, phosphorylcholine; CDP-Ch, cytidine-5' diphosphocholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol. (B) Detergent extracts of SMS2-expressing and control cells were incubated with NBD-ceramide and [3H]-choline labelled PC. The lipids were extracted, separated by two-dimensional TLC and analysed for fluorescence and radioactivity. Note that only SMS2-expressing cells synthesized NBD-SM, and that this NBD-SM was labelled with [3H]-choline.

Fig. 7. Human SMS1 and SMS2 exhibit reverse activity. Detergent extracts of yeast cells expressing human SMS1, SMS2 or transformed with empty vector (control) were incubated with NBD-diacylglycerol (NBD-DAG; 1.8  $\mu$ M) in the presence or absence of different head group donors (22  $\mu$ M), as indicated. Formation of NBD-PC was monitored by one-dimensional TLC and quantified as described in Materials and methods.

Fig. 8. Conserved sequence motifs in SMS1, SMS2 and SMSr proteins. (A) Alignment of human SMS1, SMS2 and SMSr amino acid sequences. Identical residues are highlighted in black and conservative amino acid substitutions in grey. Conserved residues within four homology motifs, designated D1 to D4, are highlighted in blue and conservative amino acid substitutions in the D2 and D4 motifs of SMSr proteins in green. Note that motifs D3 and D4 display similarity to the C2 and C3 domains in LPPs, with identical residues highlighted in red. Regions predicted to form transmembrane domains, TM1 to TM6, are marked by a black line. (B) Alignment of the four homology motifs in SMS and SMSr proteins from humans (*Hu*), *C. elegans* (*Ce*), *P. falciparum* (*Pf*) and *D. melanogaster* (*Dm*). PlasmoDB accession no. of *Pf*SMS1 and *Pf*SMS2 are MAL6P1.177 and MAL6P1.178, respectively. Putative active site residues in consensus

sequences are underlined. Symbols used are n, small neutral amino acid;  $\Phi$ , aromatic amino acid; b, branched amino acid; x, any amino acid.

**Fig. 9.** Sequence listings 1 - 11: conserved sequence motifs of  
5 phosphotransferases as indicated in figure 8 B

Sequence listings 12 - 22: polypeptide sequences of cholinephosphotransferases and ethanolaminephosphotransferases

- |    |     |  |
|----|-----|--|
| 10 | 12. | <i>human</i> sphingomyelin synthase 1                                  |
|    | 13. | <i>human</i> sphingomyelin synthase 2                                  |
|    | 14. | <i>human</i> sphingomyelin synthase related                            |
|    | 15. | <i>Caenorhabditis elegans</i> sphingomyelin synthase 1                 |
|    | 16. | <i>Caenorhabditis elegans</i> sphingomyelin synthase 2                 |
| 15 | 17. | <i>Caenorhabditis elegans</i> sphingomyelin synthase 3                 |
|    | 18. | <i>Caenorhabditis elegans</i> sphingomyelin synthase distantly related |
|    | 19. | <i>Caenorhabditis elegans</i> sphingomyelin synthase related           |
|    | 20. | <i>Plasmodium falciparum</i> sphingomyelin synthase 1                  |
|    | 21. | <i>Plasmodium falciparum</i> sphingomyelin synthase 2                  |
| 20 | 22. | <i>Drosophila melanogaster</i> sphingomyelin synthase related.         |

**Fig. 10.** Human SMS1 and SMS2 are encoded by ubiquitously expressed genes. Northern blot analysis of SMS1 and SMS2 transcripts (arrows) in various human tissues. Random prime-labelled human SMS1 or SMS2 cDNA  
25 was hybridised to a human poly(A)<sup>+</sup> RNA blot (Origene, Rockville, MD). As a control for loading, the RNA blot was stripped and rehybridized with a human  $\beta$ -actin cDNA probe. Mobilities of RNA size markers are indicated.

**Fig. 11.** Human SMS1 and SMS2 localise to different cellular  
30 organelles. (A)

HeLa cells co-transfected with V5-tagged SMS1 and *myc*-tagged sialyltransferase were incubated in the presence or absence of 10  $\mu$ M nocodazole for 60 min at 37°C, fixed, and then co-stained with rabbit anti-V5 and mouse anti-*myc* antibodies. Counterstaining was with FITC-conjugated goat anti-rabbit and Texas red-conjugated goat anti-mouse antibodies. (B) HeLa cells transfected with V5-tagged SMS2 were biotinylated on ice, fixed and then co-stained with mouse anti-V5 and rabbit anti-biotin antibodies. Counterstaining was with Texas red-conjugated goat-anti mouse and FITC-conjugated goat anti-rabbit antibodies. Bar, 10  $\mu$ m.

10

Fig.12. Human SMS2 is partially associated with the Golgi. HeLa cells co-transfected with V5-tagged SMS2 and *myc*-tagged sialyltransferase were incubated in the presence or absence of 10  $\mu$ M nocodazole for 60 min at 37°C, fixed, and then co-stained with rabbit anti-V5 and mouse anti-*myc* antibodies. Counterstaining was with FITC-conjugated goat anti-rabbit and Texas red-conjugated goat anti-mouse antibodies. Bar, 10  $\mu$ m.

15

Fig. 13. Membrane topology of human SMS1 and SMS2 proteins. (A)

Schematic

view of the predicted membrane topologies of V5-tagged SMS1 and SMS2, and the Golgi-associated type I membrane protein, p24. (B) Immunoblot of intact or lysed HeLa cells expressing V5-tagged SMS1 or SMS2 and pretreated with 8 mM trypsin for 30 min at 30°C in the presence or absence of 0.4% Triton X100, as indicated. Immunoblots were stained with antibodies against the V5 epitope ( $\alpha$ -V5) or against p24 ( $\alpha$ -p24).

25

Fig. 14. SMS1 and SMS2 are required for cell growth and survival.

(A) SMS1, SMS2 and SMSr are expressed in human HeLa cells. Open reading frames of human SMS1, SMS2 and SMSr were amplified by reverse-

transcriptase PCR on 5  $\mu$ g total RNA isolated from HeLa cells, according to

30

instructions of the manufacturer (Titan One Tube RT-PCR, cat. No. 1855476, Roche Diagnostics GmbH, Mannheim, Germany). Products were separated on a 1% agarose gel and stained with ethidium bromide. (B) HeLa cells stably transfected with V5-tagged SMS1 (SMS1-V5/pcDNA3.1) were treated with

5 lamin A, SMS1 or SMS2 siRNA duplexes for 72 hr before Western blot analysis using anti-V5 and anti-lamin A antibodies, as described (Elbashir et al., Nature 411, 494-498). Lamin A, SMS1 and SMS2 were targeted with siRNA duplexes designed from the sequences 5'-aactggacttccagaagaacatc-3', 5'-aactacactcccagctacctgg-3', and 5'-aacccaagagcttatccagtg-3', respectively. (C)

10 Hela cells stably transfected with V5-tagged SMS1 were treated with lamin A or SMS1 siRNA for 72 hr, and then fixed, permeabilized and co-stained with mouse-anti GM130 and rabbit anti-V5 antibodies. Counterstaining was with Texas red-conjugated goat-anti-mouse and FITC-conjugated goat-anti-rabbit antibodies. (D) Growth curves of Hela cells stably transfected with V5-tagged

15 SMS1 and treated with lamin A, SMS1 and/or SMS2 siRNA duplexes in serum-free Optimem I medium supplemented with Glutamax I (GIBCO/Invitrogen, Carlsbad, CA). Cell numbers were assessed at 0, 24, 48 and 72 hr after treatment with siRNA duplexes.

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Table: EST clones and primers used in this study

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Protein name	Accession number <sup>1</sup>	EST clone ID	Primer pairs N- and C-terminal (5'-3')
<i>HuCSS1α1</i>	Q9NXE2	IRAKp961F14	AAGCCATGGCTGTAGGAAACAACAC
		32 <sup>2</sup>	GGTAACTTCGGTCATGGAC
<i>HuCSS1α2</i>	Q96GM1	IRALp962F05	AAGCCATGGCGGGAGGGAGA



12

		26 <sup>2</sup>	GGTGGCCACGGCGGGC
<i>HuCSS2 α</i>	Q96SS7	IRALp962I04	AAGCCATGCCAGCTTCCCAGAG
		17 <sup>2</sup>	CCAGGCAGAGATGAGCATC
<i>HuCSS3α1</i>	Q86VZ5	LGN01891 <sup>3</sup>	AAGCCATGATCCTTGTAGGACTCTG
( <i>SMS1</i> )			TGTGTCATTACACCAGCCGG
<i>HuCSS3α2</i>	Q8NHU3	IRAKp961F14	AAGCCATGAATATGTTTGATGCTGACA
( <i>SMS2</i> )		33 <sup>2</sup>	ATCCTTATAAGCCCGTGTGG
<i>HuCSS3β</i>	Q96LT4	IMAGp958I13	AAGCCATGGCAGGTCCTAATCAACTC
		1239Q <sup>2</sup>	TCCAATTAGTCTTTTCATTATTGC
<i>MmCSS1α2</i>	Q8VCY8	IRAKp961L15	AAGCCATGGCTGGAGGGAG
		31 <sup>2</sup>	GGTGGCCACGGCGGGC
<i>MmCSS2α</i>	Q91WB2	IMAGp998B0	AAGCCATGCCAGCTTCCCAGA
		810812 <sup>2</sup>	CCAGGCAGAGATGAGCATC
<i>MmCSS3α1</i>	Q8VCQ6	IRAKp961L21	AAGCCATGAAGGAAGTGGTTTAC
( <i>SMS1</i> )		41 <sup>2</sup>	TGTGTCGTTTACCAGCCGG
<i>CeCSS1γ</i>	Q22250	YK289g7	AAGCCATGTCCGTGCCAGCTTCG
		/YK572h5 <sup>4</sup>	GTACCGATATCCATCATTTTG
<i>CeCSS3α1</i>	Q9XTV2	YK559h9 /	AAGCCATGAAAATGTCTTGGAATCATCA
( <i>SMS1</i> )		YK517e10 <sup>4</sup>	A TTTTGGCAGAGACATGGTAG
<i>CeCSS3α2</i>	Q20735	YK428e6 /	AAGCCATGACAAACAGTTCGGAGTTC
( <i>SMS2</i> )		YK109h8 <sup>4</sup>	TTGCAATTTGTAGTTGATACGA
<i>CeCSS3β</i>	Q20696	YK524b8 <sup>4</sup>	AAGCCATGCTGGATAACAGACCTATAC
			ATTGTGCTTTTTGGTATGATTTTTG

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1 Swiss-Prot/TrEMBL

2 Obtained from RZPD Deutsches Ressourcenzentrum für Genomforschung, Berlin,  
Germany

5 3 Provided by Dr. Sumio Sugano, Institute of Medical Science, University of Tokyo,  
Japan

4 Provided by Dr. Yuji Kohara, National Institute of Genetics, Mishima, Japan

To achieve the targets of the invention a complementary approach was  
10 pursued for the identification of animal SM synthase that takes advantage of  
structural information available for enzymes catalyzing analogous reactions.  
In contrast to most animal cells, plants, fungi and yeast do not produce SM.  
Instead, these organisms add phosphoinositol to phytoceramide to generate  
inositol phosphorylceramide, or IPC (Dickson, 1998). IPC production in the  
15 yeast *Saccharomyces cerevisiae* requires the product of the *AUR1* gene (Nagiec  
et al., 1997).

Sequence analysis of Aur1p proteins from different fungi revealed four  
conserved motifs (Heidler and Radding, 2000) two of which are similar to the  
C2 and C3 domains present in members of the lipid phosphate phosphatase  
20 (LPP) family (Waggoner et al., 1999).

LPPs play a critical role in cell signaling by controlling the conversion of  
bioactive lipid phosphate esters such as (lyso)phosphatidic acid and  
sphingosine-1-phosphate to their dephosphorylated counterparts. The  
conserved C1, C2 and C3 domains in LPPs likely constitute the active site for  
25 cleavage of the bond between the lipid hydroxyl and phosphate groups  
(Neuwald, 1997).

In the case of Aur1p, this reaction would represent the first step in the  
transfer of inositol phosphate from PI, with the resulting enzyme-phosphate  
intermediate being subjected to nucleophilic attack by the oxygen of ceramide

rather than the oxygen of water used by LPPs. The presence of LPP-like motifs, together with the IPC synthase activity found associated with affinity-purified Aur1p, suggests that Aur1p is directly responsible for IPC synthesis.

Insects and mammals contain an ethanolamine phosphorylceramide  
5 (EPC) synthase, another phosphotransferase, that may be structurally related to SM synthase.

The insect *Drosophila melanogaster* (*Dm*) does not generate SM, but instead produces phosphoethanolamine phosphorylceramide (EPC (Rietveld et al., (17). Moreover, it has been shown that the endoplasmic reticulum of mammalian  
10 cells harbours an EPC synthase (Malgat et al., 1987). Furthermore, the nematode *Caenorhabditis elegans* (*Ce*) generates phosphorylcholine-substituted glycolipids (Lochnit, G., et al., 2000) still another phosphotransferase, that may be structurally related to SM synthase.

Based on the above considerations a bioinformatics and functional  
15 cloning strategy was developed to identify the enzyme responsible for SM synthesis in animals and to identify other cholinephosphotransferases. The present invention provides a surprisingly successful strategy which comprises a process to isolate candidates for functional genes of a previously unidentified enzyme with known activity from a huge database by combining at least four  
20 characteristics based on data from bio-informatics and from biochemistry, viz.

- presence of a sequence motif shared with previously identified enzymes having a related function
- biochemical function of the gene should be unknown until now
- no structural homologues in an organism that does not contain the enzyme
- 25 – ability to mediate a reaction catalysed by the unidentified enzyme upon its heterologous expression in an organism or cell lacking said enzyme activity.

Preferably also the presence or non-presence of transmembrane domains depending on the working mechanism of the enzyme in relation to the membrane is considered.

By searching the human, mouse, *C. elegans* and *Drosophila* protein databases using a sequence motif shared by LPPs and Aur1p homologues and further selection on the basis of the three additional criteria mentioned above and, a set of cholinephospho transferase sequences was collected as depicted in  
5 Fig. 8B. Conserved residues within a lot of homology motifs have been found.

Therefore, the present invention further provides an isolated polypeptide comprising one or more of the amino acid motifs selected from the group consisting of a sequence of at least 80%, preferably 90 % identity to any  
10 of

(a) P-L-X-D-X(35,75)-R-R-X(8)-[YF]-X(2)-R-X(6)-T

(b) C-X-D-X(3)-S-G-H-T

15 (c) H-Y-[TS]-X-D-[VI]-X(3)-[FYI]-X(6)-F-X(2)-Y-H.

The wording isolated means that said polypeptide has been identified in a database comprising a large amount of polypeptides.

In particular said polypeptide is derived from the group consisting of  
20 *Animalia*, *Alveolata* and *Kinetoplastida*. Preferably, said polypeptide is selected from the group comprising any of the SEQ ID No. 1 - 11 or at least 70%, more preferably at least 80%, still more preferably at least 90% similarity to the conserved amino acid motifs listed therein.

Furthermore the present invention provides a polypeptide comprising an  
25 amino acid sequence with at least 70%, preferably at least 80% and still more preferably at least 90% similarity to any of the SEQ ID No. 12 - 22. Each of these sequences comprise amino acid motifs of the SEQ ID No. 1 - 11 or sequences related thereto so that the similarity grade of at least 70 % to any of the SEQ ID No. 12 - 22 will be reached.

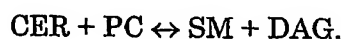
The present invention also provides a polypeptide comprising an amino acid sequence with at least 20 % identity to any of the SEQ ID No. 12 - 22. In particular, a polypeptide comprising an amino acid sequence with at least 30 % identity to SEQ ID No. 12 and therefore at least also including SEQ ID No. 13.

5        Furthermore, a polypeptide is provided by the present invention comprising an amino acid sequence with at least 40 % identity to any of the SEQ ID No. 19 - 21. Finally, the invention provides a polypeptide comprising an amino acid sequence with at least 22 % identity to SEQ ID No. 22.

10        According to a preferred embodiment of the present invention a polypeptide is provided with one or more of the activities selected from the group consisting of cholinephosphotransferases and ethanolaminephosphotransferases, preferably one or more of the activities selected from the group consisting of sphingomyelin synthase, ethanolamine phosphorylceramide synthase, phosphatidylcholine:glycoprotein cholinephosphotransferase and  
15        phosphatidylcholine:glycolipid cholinephosphotransferase. Furthermore, a nucleotide sequence selected from the group consisting of a nucleotide sequence coding for any of the amino acid sequences of a polypeptide with one or more of said activities and an anti sense nucleotide sequence that is complementary thereto is provided, together with a plasmid, a vector and an  
20        organism or micro organism and a cell line comprising said nucleotide sequences.

      According to a further preferred embodiment of the present invention a process is provided for producing sphingomyelin synthase or sphingomyelin comprising the expression of the nucleotide sequences coding for a polypeptide  
25        with sphingomyelin synthase activity in a (micro)organism or cell line comprising the nucleotide sequences coding for such a polypeptide.  
      Furthermore, the use of one of more of the nucleotide sequences coding for a polypeptide with sphingomyelin synthase activity to influence the reaction

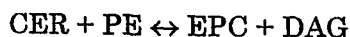
in *vivo* or *in vitro* has been provided. In this way a powerful means has been created to enhance the conversion of CER and PC into SM and DAG and vice versa. Besides this, the present invention also provides the use of one of more  
5 of the nucleotide sequences coding for a polypeptide with sphingomyelin synthase activity to identify or develop compounds influencing the reaction



10

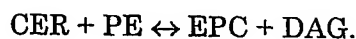
According to still a further preferred embodiment of the present invention a process is provided for producing ethanolamine phosphorylceramide synthase or ethanolamine phosphorylceramide comprising the expression of the nucleotide sequences coding for a polypeptide  
15 with ethanolamine phosphorylceramide synthase activity in a (micro)organism or cell line comprising the nucleotide sequences coding for such a polypeptide. Furthermore, the use of one of more of the nucleotide sequences coding for a polypeptide with ethanolamine phosphorylceramide activity to influence the reaction

20



in *vivo* or *in vitro* has been provided. In this way a powerful means has been created to enhance the conversion of CER and PE into EPC and DAG and vice  
25 versa. Besides this, the present invention also provides the use of one of more of the nucleotide sequences coding for a polypeptide with ethanolamine phosphorylceramide synthase activity to identify or develop compounds influencing the reaction

30



According to another preferred embodiment of the invention the application of the compounds mentioned above which can be identified or developed by the use of one of more of the nucleotide sequences coding for a polypeptide with sphingomyelin synthase and/or ethanolamine phosphorylceramide synthase in medical use, especially for the manufacture of medicaments treating a disease selected from the group consisting of cancer, metabolic diseases, for instance the Niemann Pick A disease and diseases caused by parasites, for instance malaria, is provided.

According to a further preferred embodiment of the present invention a process is provided for producing phosphatidyl:glycoprotein cholinephosphotransferase or phosphatidyl:glycolipid cholinephosphotransferase, phosphorylcholine-substituted glycoprotein or phosphorylcholine-substituted glycolipid comprising the expression of the nucleotide sequences coding for a polypeptide with phosphatidyl:glycoprotein cholinephosphotransferase or phosphatidyl:glycolipid cholinephosphotransferase activity in a (micro)organism or cell line comprising the nucleotide sequences coding for the corresponding polypeptide. "Corresponding" stands for the nucleotide sequences which code for the enzyme with the corresponding activity, so either for the enzyme with the phosphatidyl:glycoprotein cholinephosphotransferase activity or for the enzyme with the phosphatidyl:glycolipid cholinephosphotransferase activity. Furthermore, the use of one of more of the nucleotide sequences coding for a polypeptide with phosphatidyl:glycoprotein cholinephosphotransferase or phosphatidyl:glycolipid cholinephosphotransferase activity to influence the reaction



*in vivo* or *in vitro* has been provided. In this way a powerful means has been created to enhance the conversion of glycolipid/protein and PC into PC-substituted glycolipid/protein and DAG and vice versa.

Besides this, the present invention also provides the use of one of more  
5 of the nucleotide sequences coding for a polypeptide with  
phosphatidyl:glycoprotein  
cholinephosphotransferase activity or phosphatidyl:glycolipid  
cholinephosphotransferase activity to identify or develop compounds  
influencing the reaction

10

glycolipid/protein + PC  $\leftrightarrow$  PC-substituted glycolipid/protein + DAG.

According to another preferred embodiment of the invention the  
application of the compounds mentioned above which can be identified or  
15 developed by the use of one of more of the nucleotide sequences coding for a  
polypeptide with phosphatidyl:glycoprotein cholinephosphotransferase or  
phosphatidyl:glycolipid cholinephosphotransferase in medical use, especially  
for the manufacture of a medicament treating a disease caused by parasitic  
nematodes, is provided.

20

A detailed description of the various aspects of the present invention  
will be given here below.

A novel family of integral membrane proteins likely responsible for SM  
synthesis in animal cells was identified according to the process of the present  
invention. The two human members of this family, SMS1 and SMS2, were  
25 subjected to a detailed biochemical analysis and it is believed that their  
functional assignment as SM synthases is justified on the following grounds.

First, heterologous expression of SMS1 or SMS2 proves sufficient to  
support SM synthesis in the yeast *S. cerevisiae*, an organism lacking  
endogenous SM synthase activity (figures 2, 4 and 6). Second, in keeping with  
30 the enzymatic properties of mammalian SM synthase reported in the



literature, SMS1 and SMS2 function as bi-directional lipid cholinephosphotransferases capable of converting PC and ceramide to SM and DAG and vice versa (figures 6 and 7). Third, SMS1 and SMS2 localize to cellular organelles previously established as the principal sites of SM synthesis, namely the Golgi (SMS1) and the plasma membrane (SMS2), figure 11. Fourth, SMS1 and SMS2 share sequence motifs containing putative active site residues with lipid phosphate phosphatases and candidate PI:ceramide inositolphosphotransferases (figure 8). Finally, SMS1 and SMS2 adopt a membrane topology whereby the putative active site residues are facing the exoplasmic leaflet (figure 13), hence the side of the membrane where SM synthesis was found to occur.

A multiplicity of SM synthase genes appears widely spread among organisms generating SM. Human SMS1 and SMS2 are highly conserved in mammals and evidence was found for the existence of up to three functional homologues in the nematode *C. elegans*. Moreover, two orthologous sequences in the human malaria parasite *P. falciparum* were identified according to the process of the present invention, suggesting that this lower eukaryote too contains different SM synthases encoded by separate genes. SM synthesis in *P. falciparum* occurs in the Golgi apparatus as well as in the network of tubovesicular membranes (TVM) emerging from the parasitophorous vacuolar membrane during intraerythrocytic development (Elmendorf and Haldar, 1994). A differential sensitivity to 1-phenyl-2-acylamino-3-morpholino-1-propanol has been taken as evidence that the Golgi- and TVM-associated activities correspond to different enzymes (Kauer et al., 1995). Our current findings provide a novel opportunity to test this prediction and may facilitate the development of new drugs against parasites as for instance malaria.

The uniform tissue distributions of transcripts for SMS1 and SMS2 in humans (figure 10) suggest that most mammalian cell types would contain both SM synthase isoforms. Biochemical analysis of human SMS1 and SMS2 thus far revealed no fundamental differences in enzymatic properties, even

though the possibility that substrate preferences are lost when enzymes are assayed in a heterologous, detergent-containing system cannot be excluded. For now, the most striking difference between SMS1 and SMS2 concerns their subcellular localisation. While SMS1 seems to represent the well-known Golgi-associated SM synthase, SMS2 primarily resides at the plasma membrane. Hence, SMS1 would be proximal to SMS2 with respect to receiving newly synthesized ceramide from the ER. A challenging prospect is that, whereas the Golgi enzyme would be responsible for generating the bulk of cellular SM, the second enzyme may serve a principal role in signal transduction at the plasma membrane. The activation of a sphingomyelinase (SMase) and subsequent liberation of ceramide at the plasma membrane has been recognised as an important signalling event in the regulation of fundamental cellular processes that include cell cycle arrest, differentiation and apoptosis (Kolesnic and Hannun, 1999; Pettus et al., 2002; Andrieu-Abadie and Levade, 2002). By converting ceramide back to SM, plasma membrane-associated SM synthase may attenuate SMase-induced signalling. This reaction would produce as a side product DAG, which is a signalling molecule in its own right (Brose and Rosenmund, 2002).

Hence, the presence of a SM synthase at the plasma membrane complicates a proper understanding of the role of ceramide as a specific signal-transducer.

A current bottleneck for elucidating the role of SM hydrolysis in cellular signalling is the unambiguous identification of the agonist-stimulated SMase. Both neutral and acid SMases have been implicated, but their precise cellular roles in SM hydrolysis and signalling remain to be clarified (Andrieu-Abadie and Levade, 2002). Since the plasma membrane-associated SM synthase is capable of catalysing the reverse reaction of SM synthesis (van Helvoort et al., 1994), it is tempting to speculate that it may execute part of the signalling events currently attributed to ligand-induced SMase. The observation that both plasma membrane- and Golgi-associated SM synthases exhibit reverse activity brings up the question of how the directionality of these enzymes is

regulated. One possibility is that the direction of the reaction is primarily determined by the relative concentrations of ceramide and DAG in the membrane. On the other hand, given the biological importance attributed to DAG and ceramide, one may expect the interconversion of these molecules to be subjected to a more elaborate form of control. Indeed, it has been shown that activation of SM synthesis in primary astrocytes is an early event associated with the mitogenic activity of basic fibroblast growth factor (Riboni et al., 2001) and that the onset of TNF $\alpha$ -induced apoptosis in rhabdomyosarcoma cells is preceded by TNF-dependent inhibition of SM synthesis (Bourteele et al., 1998). Mammalian SMS1 contains a predicted SAM (sterile alpha motif) domain at the amino-terminus (residues 7-63 in human SMS1) that might provide a means for the enzyme to interact with regulatory proteins (Schultz et al., 1997). The present identification of a family of animal SM synthases offers unprecedented opportunities to further clarify the biological significance of SM metabolism and its regulation.

SM synthases contain four highly conserved sequence motifs (figure 8). Two of these, D3 and D4, are similar to the C2 and C3 phosphatase domains in LPPs and include the putative catalytic histidine and aspartate residues implicated in LPP-mediated hydrolysis of lipid phosphate esters (Neuwalder, 1997). This suggests a working mechanism for SM synthases in which the catalytic triad previously described for LPPs would function in a similar fashion. The reaction would proceed via the following steps: (1) binding of a two-chain choline-phospholipid (PC or SM) to a unique binding site, (2) nucleophilic attack on the lipid-phosphate ester bond by the histidine in D4 assisted by the conserved aspartate in this motif, (3) formation of a cholinephospho-histidine intermediate and release of DAG or ceramide, facilitated by the histidine in D3 acting as a base, (4) nucleophilic attack of the C1-hydroxyl of ceramide or DAG on the cholinephospho-histidine intermediate, assisted by the histidine in D3, (5) release of SM or PC from the active site to

allow another round of catalysis. This model provides a framework for site-directed mutagenesis and kinetic studies to reveal the working mechanism of SM synthases.

There is a striking parallel between animal SM synthase and IPC  
5 synthase in yeast. Like human SMS1, the yeast enzyme is Golgi localised and contains the key D4 motif in the Golgi lumen at the start of the last membrane span (Levine et al., 2000). This suggests that animal SM synthase and yeast IPC synthase may have evolved from a common ancestor. In contrast, the SM  
10 synthase recently identified from *Pseudomonas aeruginosa* (Luberto et al, 2003) lacks the key motifs of animal SM synthase and is a soluble, rather than an integral membrane protein. Hence, it appears that SM synthases arose at least twice during evolution.

Database searches using the catalytic site sequences from LPPs yielded, in addition to SM synthases, numerous other proteins containing partially  
15 conserved phosphatase motifs. In many cases, the biochemical functions of these proteins are not known.

Given the present findings, the possibility that some of these LPP-like proteins do not serve as phospholipases but instead catalyse novel kinds of synthetic or transphosphatidylation reactions deserves consideration. For  
20 example, mammals and insects synthesize ethanolamine phosphorylceramide, a sphingolipid analogous to SM (Malgat et al., 1986; Rietveld et al., 1999). Whether production of EPC and SM involves a single transferase that accepts both PC and PE as substrates or requires two separate enzymes has remained an open issue. However,  
25 *Drosophila* lacks SM and generates only EPC (Rietveld et al., 1999). Our finding that *Drosophila* contains an SMS-related protein, but no SMS proteins, strongly suggests that the SMS-related proteins described in this study function as dedicated EPC synthases. This possibility is currently under further investigation.

Phosphorylcholine (PC), a small haptenic molecule, is found in a wide variety of prokaryotic organisms, i. e. bacteria, and in eukaryotic parasites such as nematodes, as well as in fungi. Linked to parasite-specific glycoprotein glycans or glycolipids, it is assumed to be responsible for a variety of immunological effects, including invasion mechanisms and long-term persistence of parasites within the host. Numerous reports have indicated various effects of PC-substituted molecules derived from parasitic nematodes on signal transduction pathways in B and T lymphocytes, displaying a highly adapted and profound modulation of the immune system by these parasites.

The Nematoda, comprising parasitic and free-living species (e.g. *C. elegans*), can be regarded as promising prototypic systems for structural analyses, immunological studies and biosynthetic investigations. In this context, *Ascaris suum*, the pig parasitic nematode, is an ideal organism for immunological studies and an excellent source for obtaining large amounts of PC-substituted (macro)molecules. *C. elegans*, as a completely genome-sequenced species and expressing parasite analogous PC-substituted structures, together with the possibility for easy *in vitro* cultivation, represents a conceptual model for biosynthetic studies, whereas filarial parasites represent important model systems for human pathogens, especially in developing countries. Current knowledge on the tissue-specific expression of PC epitopes, structural data of glycoprotein glycans and glycosphingolipids bearing this substituent and biological implications for the immune systems of the respective hosts are reviewed by Lochnit et al., 2000. Since the elucidated structures of these compounds are clearly different from those present in the host, the biosynthesis of nematode-specific, PC-substituted compounds would be a promising target for the development of new anthelmintic strategies.

In the present invention it has been shown that modulating the activity of an enzyme of the group of enzymes identified as sphingomyelin synthases, ethanolamine phosphorylceramide synthases,

phosphatidylcholine:glycoprotein cholinephosphotransferase and phosphatidylcholine:glycolipid cholinephosphotransferase (further referred to as the group of enzymes) in a cell has effects on cell growth and/or survival. By lowering the activity in cells, the cells respond by a reduction in cell growth, increased sensitivity toward apoptotic stimuli and/or reduced cell survival. Upregulation of the activity induces cell growth, rejuvenation of the cells and/or a reduced sensitivity toward apoptotic signals. An effect is, of course preferably relative to the behaviour of normal cells. These cells typically have effective but not saturating levels of an activity of an enzyme of the group of enzymes and preferably of a sphingomyelin synthase. The possibility of detecting a change in the activity of an enzyme of the group, that can be correlated to a particular amino acid sequence has led the inventors to methods for determining whether a compound is capable of modulating an enzymatic activity displayed by a cell, said activity comprising an activity of an enzyme of the group of enzymes identified as sphingomyelin synthases, ethanolamine phosphorylceramide synthases, phosphatidylcholine:glycoprotein cholinephosphotransferase and phosphatidylcholine:glycolipid cholinephosphotransferase, said method comprising providing said cell with a nucleic acid encoding a polypeptide according to the invention, contacting said cell with said compound and determining whether said enzymatic activity is modulated. In a preferred embodiment said method comprises detecting whether said activity is inhibited. Thus the invention further provides the use of an inhibitor of a enzyme activity of the group, preferably a sphingomyelin synthase of the invention, as a cell death promoter. Preferably said cell is a parasitic cell or a mammalian tumor cell, preferably a human tumor cell. The compound capable of modulating said enzyme activity displayed by the cell, can act on the level of expression of the enzyme, wherein a higher expression results in upregulation of the enzyme activity displayed by the cell and wherein a lower expression results in downregulation of the enzyme activity displayed by the cell.

Alternatively, the compound can act on the activity of the enzyme in the cell, i.e. the compound can increase the activity of the enzyme and thereby upregulate the enzymatic activity displayed by the cell, or the compound can decrease the activity of the enzyme and thereby downregulate the enzymatic activity displayed by the cell.

In a preferred embodiment of a method of the invention, said enzyme activity of the group comprises a sphingomyelin synthase activity. A polypeptide of the invention comprising sphingomyelin synthase activity preferably comprises an amino acid sequence having at least 80% and preferably at least 90% identity to an amino acid sequence according to formula (a), (b) or (c). In a preferred embodiment said polypeptide of the invention comprising sphingomyelin synthase activity preferably comprises an amino acid sequence having at least 80% and preferably at least 90% identity to an amino acid sequence according to formula (a), (b) and (c). In a particularly preferred embodiment a polypeptide of the invention comprising sphingomyelin synthase activity in a mammalian cell (a mammalian sphingomyelin synthase) and having the above mentioned identity to the formulas, further comprises at least 50% and preferably at least 90% sequence identity with huSMS1 (Seq. ID 12) or huSMS2 (Seq. ID 13) depicted in figure 8. In a particularly preferred embodiment a polypeptide of the invention comprising sphingomyelin synthase activity in a nematode cell (a nematode sphingomyelin synthase) and having the above mentioned identity to the formulas, further comprises at least 50% and preferably at least 90% sequence identity with ceSMS1 (Seq. ID 14), ceSMS2 (Seq. ID 15) or ceSMS2 (Seq. ID 16) depicted in figure 8. A polypeptide of the invention comprising sphingomyelin synthase activity in a plasmodium cell (a plasmodium sphingomyelin synthase) and having the above mentioned identity to the formulas, preferably further comprises at least 30% and preferably at least 90% sequence identity with pfSMS1 (Seq. ID 17) or pfSMS2 (Seq. ID 18) depicted in figure 8. In a particularly preferred

embodiment a polypeptide of the invention comprising ethanolamin phosphorylceramid synthase activity in a mammalian cell (a mammalian ethanolamin phosphorylceramid synthase) and having the above mentioned identity to the formulas, further comprises at least 50% and preferably at least 90%, sequence identity with huSMSr (Seq. ID 19). In a particularly preferred embodiment a polypeptide of the invention comprising ethanolamin phosphorylceramid synthase activity in a nematode cell (a nematode ethanolamin phosphorylceramid synthase) and having the above mentioned identity to the formulas, further comprises at least 50% and preferably at least 90% sequence identity with ceSMSr (Seq. ID 21) depicted in figure 8. A polypeptide of the invention comprising ethanolamin phosphorylceramid synthase activity in an insect cell (an insect ethanolamin phosphorylceramid synthase) and having the above mentioned identity to the formulas, preferably further comprises at least 50% and preferably at least 90% sequence identity with dmSMSr (Seq. ID 20) depicted in figure 8. A polypeptide of the invention comprising PC:glycolipid/glycoprotein cholinephosphotransferase activity in a nematode cell (an nematode PC:glycolipid/glycoprotein cholinephosphotransferase) and having the above mentioned identity to the formulas, preferably further comprises at least 30% and preferably at least 90%, sequence identity with ceSMSdr (Seq. ID 22) depicted in figure 8.

In one embodiment of the invention a polypeptide of the invention comprising sphingomyelin synthase activity preferably comprises an amino acid sequence having at least 80% and preferably at least 90% identity to an amino acid sequence according to formula (a), (b) or (c). In a preferred embodiment said polypeptide of the invention comprising sphingomyelin synthase activity preferably comprises an amino acid sequence having at least 80% and preferably at least 90% identity to an amino acid sequence according to formula (a), (b) and (c). In a particularly preferred embodiment a mammalian polypeptide of the invention comprising sphingomyelin synthase activity and having the above mentioned identity to the formulas further



comprises at least 50% and preferably at least 90%, sequence identity with huSMS1 (Seq. ID 12) or huSMS2 (Seq. ID 13) depicted in figure 8.

By providing a cell with a polypeptide of the invention having an  
5 enzyme activity of the group one produces a cell having more activity of said enzyme than before. This allows for early and easier detection of the effect that compounds have on the activity displayed by the cell. In a preferred embodiment a polypeptide of the invention having activity of an enzyme of the group is provided to a cell that did not contain this activity in detectable  
10 amounts. Such cells may be generated by inactivating the endogenous genes coding for the polypeptide or cells can be used from species and/or genera that do not contain this activity. Non-limiting examples of such species and/or genera in case of sphingomyelin synthase activity are: drosophila and yeast. Thus in a preferred embodiment the method utilizes a cell that was deficient in  
15 an enzyme activity of the group, which is preferably a sphingomyelin synthase activity, prior to providing the cell with the polypeptide of the invention.

A cell can be any type of cell. A cell can be part of an organism, where one or more of the cells of the organism are provided with (nucleic acid encoding) said  
20 polypeptide. The cell can also be a cell of a micro-organism. The micro-organism can be bacterial and is preferably a eukaryotic micro-organism. Yeasts and fungal micro-organisms are preferred.

A polypeptide of the invention preferably comprises a sequence as depicted in  
25 figure 8 or a functional part, derivative and/or homologue thereof. A functional part, derivative or homologue of a sequence as depicted in figure 8 comprises at least the same enzyme activity in kind not necessarily in amount. A functional part preferably comprises at least 80% of the amino sequence of a sequence as depicted in figure 8. A functional derivative preferably comprises  
30 not more than 5 and preferably not more than 3 amino acid substitutions

compared to a sequence of figure 8. In a preferred embodiment said substitutions are conservative substitutions. A derivative can also comprises one or more deletions and/or insertions of amino acids when compared with a sequence as depicted in figure 8. A functional homologue is a sequence derived from a different species as the species depicted in figure 8, but wherein said homologue shares a common ancestor. A functional homologue preferably comprises an amino acid sequence of formula (a), (b) and (c). In a preferred embodiment said polypeptide comprises a sequence as depicted in figure 8A or a functional part, derivative and/or homologue thereof. In one preferred aspect of the invention said polypeptide is derived from a plasmodium. Preferably having an amino acid sequence as depicted in figure 8B or a functional part, derivative and/or homologue thereof.

The compound can be any chemical or biological compound. In one embodiment said compound has an effect on expression of a polypeptide of the invention in a cell. Such a compound can be an expression cassette comprises an expressible coding region for said polypeptide. It can also comprise for instance a compound capable of modulating the activity of a promoter for the gene encoding said polypeptide in said cell. In a preferred embodiment said compound is capable of preventing translation of mRNA encoding said polypeptide in said cell. Translation is preferably inhibited through anti-sense RNA or RNAi. In a particularly preferred embodiment translation is inhibited through RNAi. RNAi is a collective term for a response of cells to double stranded RNA. A variety of different types of compounds are able to induce the specific degradation of mRNAs. None of those different types of compounds are herein excluded. In a preferred embodiment the compound comprises siRNA (Elbashir et al., Nature 411, 494-498) or a hairpin RNA. A hairpin RNA can be introduced directly into the cell or be expressed by a gene encoding the hairpin.

In another aspect the invention provides the use of a nucleic acid encoding a polypeptide according to the invention, as a probe. In a preferred aspect the invention provides the use of an oligonucleotide specific for a nucleic acid sequence encoding a polypeptide as depicted in figure 8 or a functional part, derivative and/or homologue thereof, for detecting said sequence.

Detection of the sequence can be for any purpose and is preferably for the purpose of assessing whether a cell comprises an enzyme activity of the group of enzymes mentioned above, preferably sphingomyelin synthase activity.

In another aspect the invention provides use of a nucleic acid encoding a polypeptide of the invention, preferably comprising a nucleic acid sequence encoding a polypeptide as depicted in figure 8 or a functional part, derivative and/or homologue thereof for enhancing cell survival and/or cell growth. It has been found that increasing the enzymatic activity displayed by a cell of an enzyme of the group of enzymes, has the effect that the secretion pathway of the cell is stimulated. This in yet another aspect the invention provides a method for at least in part improving the yield of an secretion product of a cell comprising providing said cell with a polypeptide of the invention, or a nucleic acid such a polypeptide, preferably comprising a nucleic acid sequence encoding a polypeptide as depicted in figure 8 or a functional part, derivative and/or homologue thereof. In a preferred embodiment said polypeptide comprises sphingomyelin synthase activity. In this embodiment said cell is preferably a mammalian cell or a cell of a eukaryotic micro-organism.

A method for determining whether a compound is capable of modulating an enzymatic activity displayed by a cell, said activity comprising an activity of an enzyme of the group of enzymes identified as sphingomyelin synthases, ethanolamine phosphorylceramide synthases,

phosphatidylcholine:glycoprotein cholinephosphotransferase and phosphatidylcholine:glycolipid cholinephosphotransferase can be performed using any method for the detection of said enzymatic activity. In a preferred embodiment the method for detecting said enzyme activity comprises  
5 providing said cell or a fraction thereof with a labelled substrate for said enzyme. Said substrate is preferably a substrate for sphingomyelin synthase. The method preferably further comprises harvesting sphingolipid from said cell or said fraction and detecting labelled sphingolipid. Detection of said labelled sphingolipid is preferably done using (thin layer) chromatography or  
10 mass spectrometry.

Polypeptides of the invention are associated with a membrane of the cell. The localization varies with the particular polypeptide. It has been observed that this property can be used to generate chimeric polypeptides having sequences from at least two polypeptides of the invention which have  
15 an altered distribution in the cell when compared to at least one of the polypeptides said chimeric polypeptide is derived from. Such chimeric polypeptides and nucleic acids encoding the chimeric polypeptides are therefore also included in the invention. Thus the invention provides a method for targeting a first polypeptide according to the invention to a different  
20 cellular compartment comprising providing a cytosolic part of said first polypeptide with a cellular compartment localization signal of a cytosolic part of a second polypeptide according to the invention, wherein said first and said second polypeptide, when unmodified, reside in different cellular compartments. Chimeric polypeptides wherein the at least two original  
25 polypeptides reside in essentially the same cellular compartment are also included in the invention. It is also possible to attach known cellular localization signals to a polypeptide of the invention. Such known cellular localization signals, for instance, an endoplasmatic reticulum retention signal do not have to be derived from a polypeptide of the invention. The cellular  
30 localization signal is preferably present in the cytosolic C-terminal end of a

polypeptide of the invention. Thus preferably the cytosolic C-terminal end of said first polypeptide is provided with the with a cellular compartment localization signal of a cytosolic part of the second polypeptide. Preferably said cytosolic part of said second polypeptide comprises the C-terminal end of a polypeptide of the invention. Another way to target a polypeptide of the invention to a different compartment is to remove the cellular localization signal from the cytosolic part (preferably the C-terminal cytosolic part) of the polypeptide.

The polypeptide can be targeted to any cellular compartment having a membrane and be active there. In a preferred embodiment said cellular compartment comprises a plasma membrane, an endosomal compartment, a Golgi, an endoplasmatic reticulum or a combination thereof. Cellular localization signals do not have to be absolute in that there can be cases wherein detectable amounts of polypeptide are spread over at least two cellular compartments. A polypeptide is said to be targeted to a different compartment when, compared to the original cellular localization of one of the polypeptides of the invention, the chimeric polypeptide is detectable in at least one other compartment or is not detectable in a compartment where one of the single polypeptides is detectable. A special case of targeting is where not the localization but rather the relative distribution over compartments changes. For instance, when an percentage X of a polypeptide of the invention is normally present in a first compartment and a percentage Y is present in a second compartment. In this example targeting is said to have taken place when the percentage in at least one of said at least two compartments is altered by more than 30%. Preferably, the change is reciprocal.

Considering that the cellular compartment localization signal of a polypeptide of the invention is predominantly determined by a C-terminal cytosolic part of said polypeptide it is preferred that said cellular compartment localization signal of the C-terminal cytosolic part of said second polypeptide replace the C-terminal cytosolic part of said first polypeptide.

It will be clear to the skilled in the art that by the present invention yielding purified cholinephosphotransferases and ethanolaminephosphotransferases a new field of development for new compounds with valuable properties as for instance drugs has been made available. The present invention will further be elucidated by the following examples without in any way restricting the broad scope thereof.

### Materials and methods

#### *Chemicals*

Sphingosyl-(NBD-hexanoyl)-phosphocholine (NBD-SM) and (NBD-hexanoyl)-ceramide (NBD-Cer) were from Molecular Probes (Eugene, OR) and Oleoyl-(NBD-hexanoyl)-phosphocholine (NBD-PC) from Avanti Polar Lipids (Alabaster, AL). [methyl-<sup>14</sup>C]-choline was from ICN Biomedicals (Irvine, CA) and L-3-phosphatidyl [N-methyl-<sup>3</sup>H]-choline, 1,2-dipalmitoyl from Amersham Pharmacia (Piscataway, NJ). NBD-DAG was produced from NBD-PC by treatment with phospholipase C from *B. cereus* as described (Trotter, 2000). Egg L- $\alpha$ -phosphatidylcholine, lyso-phosphatidylcholine, L- $\alpha$ -phosphatidylethanolamine, L- $\alpha$ -phosphatidylglycerol, L- $\alpha$ -phosphatidylserine, soybean L- $\alpha$ -phosphatidylinositol, dioctanoyl L- $\alpha$ -phosphatidic acid, SM (from bovine brain), choline, phosphocholine, cytidine 5'-diphosphocholine, O-tricyclo[5.2.1.0<sup>2,6</sup>]dec-9-yl dithiocarbonate potassium salt (D609), N-ethylmaleimide (NEM) and phospholipase C (*Bacillus cereus*) were from Sigma Aldrich (St. Louis, MO).

*Selection, cloning and expression of CSS sequences*

Animal entries in the Swiss-Prot/TrEMBL protein sequence database were searched using Prosite (<http://www.expasy.org/tools/scanprosite>) against sequence motif H-[YFWH]-X<sub>2</sub>-D-[VLI]-X<sub>2</sub>-[GA]-X<sub>3</sub>-[GSTA] and then further  
5 selected based on three additional criteria as described under Results. Candidate SM synthases (CSS) were assembled into different groups by multiple sequence alignments and phylogenetic trees generated with ClustalW and PHYLIP. Open reading frames (ORFs) in selected CSS sequences were PCR amplified using Taq polymerase (MBI Fermentas, Hanover, MD)  
10 according to information provided in Table I. PCR products were cloned into yeast expression vector pYES2.1/V5-His-TOPO (Invitrogen Corporation, Carlsbad, CA) and the resulting plasmids used to transform *S. cerevisiae* strain IAY11 (MAT $\alpha$  ura3-52 his3 $\Delta$ 200 leu2-3, -112 trp $\Delta$ 901 ade2-101 ade3- $\Delta$ 853, provided by Ian Adams, MRC-LMB, Cambridge, UK). Transformants  
15 were grown in complete minimal uracil dropout medium containing 2% (w/v) galactose. Expression of CSS proteins was verified by Western blot analysis with mouse anti-V5 antibodies (Invitrogen). PCR amplified ORFs of human SMS1 and SMS2 were cloned into mammalian expression vector pcDNA3.1/V5-His-TOPO (Invitrogen) and the resulting plasmids (SMS1-  
20 V5/pcDNA3.1 and SMS2-V5/pcDNA3.1) used to transfect HeLa cells.

*SM synthase assay on cell lysates*

CSS-expressing yeast cells were lysed at 100 OD<sub>600</sub>/ml by bead bashing  
25 at 4 °C in RB1 buffer (120 mM K-glutamate, 15 mM KCl, 5 mM NaCl, 2 mM MnCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 20 mM Hepes-KOH, pH 7.2) containing freshly added protease inhibitors (Holthuis et al., (1998).

Lysates were centrifuged at 700 g for 10 min at 4 °C and supernatants (0.25 ml per reaction) preincubated with NEM (1 mM) for 10 min at 37 °C. The  
30 reaction was initiated by addition of 5 nmol of NBD-Cer in 0.1 ml RB1 buffer

and incubation continued for 15 min. Lipids were extracted by addition of 1.7 ml chloroform:methanol (1:2.2), dried under N<sub>2</sub> and subjected to butanol/water partitioning. Lipids recovered from the butanol phase were separated by one-dimensional TLC for 30 min in solvent I: chloroform/methanol/4.2 M NH<sub>4</sub>OH (9:7:3, v/v), followed by 30 min in solvent II: chloroform/methanol/acetic acid (45:30:7, v/v). Fluorescent images were recorded on a STORM 860 Imaging Analysis System (Molecular Dynamics, Sunnyvale, CA) and analysed with ImageQuant software.

#### 10 *Metabolic labelling*

CSS-expressing yeast cells (0.5 OD<sub>600</sub>) were inoculated in 5 ml medium containing 10 µCi [*methyl*-<sup>14</sup>C]-choline and 10 nmol NBD-Cer. Cells were grown for 16 h at 30 °C, washed in water, and lipids were extracted by bead bashing in H<sub>2</sub>O/methanol/chloroform (5:16:16, v/v). The organic extracts were dried under N<sub>2</sub>, subjected to butanol/water partitioning, and lipids recovered from the butanol phase were deacylated by mild base treatment using 0.2 N NaOH in methanol (60 min, 30 °C). After neutralizing with 1 M acetic acid, lipids were extracted with chloroform and separated by two-dimensional TLC using solvent I in the first, and solvent II in the second dimension. Radio-labelled lipids were detected by exposure to BAS-MS imaging screens (Fuji Photo Film Co., Japan) and read out on a BIO-RAD Personal Molecular Imager.

#### 25 *Preparation of detergent extracts*

SMS1/SMS2-expressing yeast cells were lysed at 100 OD<sub>600</sub>/ml by bead bashing at 4 °C in lysis buffer (50 mM Tris-HCl, pH 7.0, 1 mM EDTA, 0.3 M sucrose) containing protease inhibitors. Post nuclear supernatants were prepared as above and loaded onto a 60% (w/w) sucrose cushion and then



centrifuged at 100.000 g for 60 min at 4 ° C. Membranes derived from  $\pm$  500 OD<sub>600</sub> of cells were resuspended in 1 ml ice-cold extraction buffer (50 mM Tris-HCl, pH 7.0, 10% glycerol, 1% Triton X-100, 1 mM MnCl<sub>2</sub>) containing protease inhibitors, incubated for 60 min at 4 ° C while rotating and then centrifuged at 100.000 g for 60 min at 4 ° C. Detergent extracts were diluted to a final protein concentration of 1.5 mg/ml in extraction buffer and 100  $\mu$ l aliquots were snap frozen in liquid N<sub>2</sub> and stored at -80 ° C.

*SM synthase and reverse transferase assays on detergent extracts*

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Detergent extracts were diluted 5- to 8-fold in extraction buffer, and 50  $\mu$ l mixed with 5  $\mu$ l of a 1.2 mM head group donor stock (e.g. CDP-choline, PC, PI) or with 15  $\mu$ Ci (350-700 fmol) of [<sup>3</sup>H]-choline-containing PC prepared in extraction buffer. Following a 15 min preincubation at 37 ° C, reactions were started by addition of 220  $\mu$ l RB2 buffer (50 mM Tris-HCl, pH 7.0, 5 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 1 mM NEM) containing 5 nmol NBD-ceramide (SM synthase assay) or 5 nmol NBD-DAG (reverse transferase assay) and incubated for 60 min at 37 ° C. Lipids were extracted, separated by TLC, and analysed as above.

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*Mass spectrometry*

Detergent extracts (400  $\mu$ l) were mixed with bovine brain ceramide (180  $\mu$ M) and egg PC (500  $\mu$ M) in a total volume of 500  $\mu$ l. After 15 min at 37 ° C, reactions were diluted 4-fold in RB2 buffer and then incubated for another 60 min at 37 ° C. Lipids were extracted in chloroform:methanol (1:2.2) and subjected to butanol/water partitioning. Residual Triton X-100 was removed by solid phase extraction on Si-60 columns (500 mg stationary phase) in acetone. Phospholipids were eluted in methanol/chloroform (19:1, v/v), dried under N<sub>2</sub>, dissolved in methanol/chloroform (2:1, v/v) and then separated by HPLC on

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two 250 x 4.6 mm Lichrosphere RP-18 end-capped columns (Merck, Darmstadt, Germany) in series (Brouwers et al., 1998). The column effluent was split in a 10:1 ratio, with the smaller fraction going to the mass spectrometer. Mass spectrometry was performed on a Sciex API-365 triple quadrupole mass spectrometer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Positive ions were generated by a turbo-ion spray ionisation source operating at +5.5 kV ionisation potential. N<sub>2</sub> (2 l/min) was used as drying gas at a temperature of 350 °C. The declustering potential (cone voltage) was set to 45V and the focus potential to 220V. Mass spectra from mass to charge ratio (m/z) 650 amu to 950 amu were recorded at a speed of 120 amu/s.

*Mammalian cell transfection and immunofluorescence microscopy*

HeLa cells were cultured in DMEM medium containing 10% fetal calf serum. Cells were grown on glass coverslips to 40% confluence and then transfected with human SMS1-V5/pcDNA3.1, SMS2-V5/pcDNA3.1 and myc-tagged sialyltransferase/pCB7 constructs using Lipofectamine 2000 (Invitrogen). After 48 h, cells were fixed in 3% paraformaldehyde/PBS and processed for immunofluorescence microscopy (see Supplementary materials for details). For cell surface biotinylation, cells were incubated twice with 0.5 mg/ml sulfo-NHS-SS-biotin (Pierce) in PBS for 20 min on ice, quenched in 10 mM glycine/PBS for 20 min and then washed in PBS prior to fixation. Immunostaining was with rabbit anti-V5 antibodies (Sigma), mouse anti-V5 antibodies (Invitrogen), mouse 9E10 anti-myc antibody (SanverTech, CA), rabbit anti-biotin antibodies (Rockland, Gilbertsville, PA), FITC- or Texas Red-conjugated goat anti-rabbit and goat anti-mouse antibodies (Jackson Laboratories, West Grove, PA). Images were obtained using a Nikon D-eclipse C1 confocal microscope.

*Protease protection assay*

HeLa cells transfected with human SMS1-V5 or SMS2-V5 constructs were washed, scraped and then lysed in ice-cold PNS buffer (120 mM K-glutamate, 15 mM KCl, 5 mM NaCl, 2 mM MnCl<sub>2</sub>, 0.8 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 1.6 mM EDTA and 20 mM Hepes-KOH, pH 7.2) by 20 passages through a 26<sup>3</sup>/<sub>4</sub> gauge needle. The homogenate was treated with 8 mM trypsin (Sigma Aldrich) in the presence or absence of 0.4% Triton X-100 for 30 min at 37 °C. Alternatively, cells were only washed and then trypsin-treated in PNS buffer as above. Homogenates and cell suspensions were transferred on ice, trypsin inhibitor (Sigma Aldrich) was added to 160 mM and samples were processed for Western blot analysis using mouse anti-V5 antibodies and rabbit anti-p24 protein (Gommel et al., 1999, provided by B. J. Helms, Faculty of Veterinary Medicine, Utrecht).

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*SDS-PAGE and Western Blot Analysis*

Protein extracts were prepared in SDS/Urea sample buffer (20 mM Tris-HCl, pH 6.8, 1 mM EDTA, 4.5 M urea, 2.5% SDS, 0.01% bromophenol blue) containing fresh protease inhibitors (Holthuis et al., (1998). Extracts were heated for 10 min at 50 °C, centrifuged briefly at 14,000 g and then resolved by SDS-PAGE on 10% polyacrylamide mini-gels. For Western blotting, nitrocellulose transfers were blocked for 90 min in PBS, 5% Protifar (Nutricia, Zoetermeer, The Netherlands), 0.2% Tween-20 (Blotto). Primary antibody incubations were performed for 1 h in Blotto. Detection was with horseradish

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peroxidase-conjugated goat anti-rabbit or anti-mouse IgG, using enhanced chemiluminescence (Amersham Pharmacia Biotech).

*Immunofluorescence Microscopy*

HeLa cells were grown on coverslips, transfected with human SMS1-V5/pcDNA3.1, SMS2-V5/pcDNA3.1 and *myc*-tagged sialyltransferase/pCB7  
5 (Sprong et al., 2001). 48 h after transfection, cells were fixed in 3% paraformaldehyde/PBS for 30 min at room temperature, quenched in PBS containing 50 mM NH<sub>4</sub>Cl, and then blocked and permeabilized for 1 h in PBS containing 0.5% BSA, 0.1% saponine (blocking buffer). Cells were labelled with mixtures of primary antibodies (1:200) for 1 h in blocking buffer, washed for 45  
10 min in blocking buffer with three buffer changes, and then incubated for 20 min with 10% goat serum in blocking buffer. For immunostaining of intact cells, incubation with primary antibodies occurred before fixation and was performed for 1 h at 4°C in PBS containing 0.5% BSA. Cells were counterstained with fluorescently labelled secondary goat antibodies (1:75) for  
15 30 min in blocking buffer, washed in blocking buffer for 45 min with three buffer changes, and then rinsed briefly in PBS and water. Mounting was in Mowiol 4-88 (Calbiochem, La Jolla, CA) containing 2.5% 1,4-diazabicyclo[2.2.2.]octane (Sigma).

### Example 1

#### Identification of candidate SM synthases from the animal database

Figure 1A shows an outline of the bioinformatics approach used to  
5 identify candidate sequences for SM synthases from the animal database.  
Candidate SM synthases were identified based on the following criteria: 1)  
presence of a sequence motif, H-[YFWH]-X<sub>2</sub>-D-[VLI]-X<sub>2</sub>-[GA]-X<sub>3</sub>-[GSTA],  
shared by previously characterised LPPs and Aur1p homologues; 2)  
biochemical function should be unknown; 3) no structural homologues in *S.*  
10 *cerevisiae*, since this organism lacks SM; 4) presence of multiple (>2)  
transmembrane domains, since the enzyme mechanism is intramembraneous  
and because LPPs and Aur1p proteins have 6 predicted membrane spans.  
Sequences conforming to all 4 criteria were subsequently used as queries in a  
BLAST search to track down homologous sequences that were missed in the  
15 initial search due to deviations in the LPP/Aur1p motif. This approach yielded  
9 human, 9 mouse and 9 *C. elegans* sequences that could be grouped into three  
major protein families, designated CSS1, CSS2 and CSS3 (for candidate SM  
synthase family 1-3; Figure 1B, C). Except for the presence of a common  
sequence motif, CSS proteins displayed no significant sequence similarity to  
20 Aur1p proteins or to LPP family members with known biochemical functions.  
However, human CSS1β1 is identical to PRG1, a neuron-specific candidate  
phosphatidic acid phosphatase with a role in axon growth and regenerative  
sprouting (Brauer et al., 2003)  
Database accession numbers of CSS proteins are listed in the legend of Figure  
25 1.

## Example 2

## A subset of CSS3 family members displays SM synthase activity

5 To investigate whether any of the three CSS families indeed contained SM synthases, the open reading frames of human, mouse and *C. elegans* members for which full-length cDNAs could be obtained were cloned into a yeast multicopy, GAL1 promotor plasmid in frame with a carboxy-terminal V5 epitope. The resulting plasmids were used to transform wild-type yeast and  
10 the transformants were shifted to galactose-containing medium to induce expression of recombinant proteins. Expression of proteins was verified by Western blot analysis using anti-V5 antibodies (Figure 2A). Thus, 13 of the 27 selected CSS sequences were expressed and analysed for SM synthase activity (marked by asterisks, Figure 1C). To this end, yeast cells expressing CSS  
15 protein were lysed and incubated with fluorescent C<sub>6</sub>-NBD-ceramide (NBD-Cer), a known substrate of mammalian SM synthase. Incubations were performed in the presence of N-ethylmaleimide (NEM), a potent inhibitor of yeast-associated SMases that does not affect mammalian SM synthase (Figure 3). Reaction mixtures were next subjected to one-phase lipid extraction and the  
20 lipids separated by one-dimensional TLC.

Figure 2B shows that in lysates of control cells NBD-Cer was converted exclusively into NBD-IPC. The same was true for lysates of cells expressing members of the CSS1 and CSS2 protein families. However, in lysates of cells expressing human CSS3 $\alpha$ 1, human CSS3 $\alpha$ 2, *C. elegans* CSS3 $\alpha$ 1 or *C. elegans*  
25 CSS3 $\alpha$ 2, NBD-Cer was converted to a second product with an R<sub>f</sub> value identical to that of NBD-SM. The identity of this product as NBD-SM was confirmed by electrospray ionisation tandem mass spectrometry (ESI-MS/MS; data not shown). Moreover, metabolic labelling of cells expressing human CSS3 $\alpha$ 1 or CSS3 $\alpha$ 2 with [<sup>14</sup>C]-choline in the presence of NBD-Cer resulted in  
30 the production of radio-labelled NBD-SM (Figure 2C). When NBD-Cer was

omitted, we noticed that CSS3 $\alpha$ 2-expressing cells generated small amounts of [14C]-choline-labelled lipids that were absent in control cells and distinct from PC and lysoPC. In addition, these lipids were resistant to mild alkaline hydrolysis, suggesting that they represent phytoceramide-based SMs (phytoSM; Figure 2D). Importantly, ESI-MS revealed that bovine brain ceramides incubated with detergent extracts of CSS3 $\alpha$ 2-expressing cells, but not control cells, are converted to SM (Figure 4). Together, these results indicate that CSS3 $\alpha$  proteins do not only use NBD-Cer, but also recognise naturally occurring ceramides as substrates for SM synthesis.

Human CSS3 $\alpha$ 1 and CSS3 $\alpha$ 2 share 57% sequence identity and are highly conserved in mammals (human – mouse > 90%). Based on the results presented herein, we propose to rename these proteins SMS1 and SMS2, respectively. *C. elegans* CSS3 $\alpha$ 1 (ceSMS1) and CSS3 $\alpha$ 2 (ceSMS2) share 22-27% sequence identity with human SMS1 and SMS2, and apparently represent their functional counterparts in the nematode (Figure 2B). *C. elegans* contains a third, CSS3 $\alpha$ -related protein, termed ceCSS3 $\alpha$ 3. Although we have not tested this protein for SM synthase activity, the finding implies that *C. elegans* is equipped with three independent SM synthases (Figure 1C). BLAST searches for orthologous sequences identified two proteins in the human malaria parasite *Plasmodium falciparum* (PlasmoDB accession no. MAL6P1.177 and MAL6P1.178; see also below). Hence, a multiplicity of SM synthase genes appears a general feature of organisms generating SM.

Apart from CSS3 $\alpha$ /SMS proteins, the CSS3 family contains a second cluster of CSS3 $\beta$  or SMS-related (SMSr) proteins with members in humans, mice, *C. elegans*, and *Drosophila* (Figure 1C). Human SMSr is highly conserved in mammals (human-mouse: 95%), shares > 40% sequence identity with its orthologues in *C. elegans* and *Drosophila*, and is about 34% identical to human SMS1 and SMS2. Heterologous expression of human or *C. elegans* SMSr did not yield any detectable SM synthase activity (Figure 2B, right panel). The *C. elegans* CSS3 $\gamma$  protein, renamed SMSdr, is only distantly



related to SMS and SMSr proteins (<22% identical; see also Figure 1C) and its ability to mediate SM synthesis was not tested.

### Example 3

- 5 Human SMS1 and SMS2 function as PC:ceramide cholinephosphotransferases  
with reverse activity

SM synthesis in animals proceeds by the liberation of phosphorylcholine from PC and its subsequent transfer onto the primary hydroxyl of ceramide. To  
10 establish whether human SMS1 and SMS2 function as PC:ceramide cholinephosphotransferases, their enzymatic characteristics were analysed in more detail. Hence, different donors of choline-P were tested as substrates for SM synthesis. To this end, detergent extracts of cells expressing human SMS1 or SMS2 were incubated with NBD-Cer and the formation of NBD-SM was  
15 monitored by TLC. A dilution of extracts proved necessary to render SMS-mediated synthesis of NBD-SM dependent on externally added head group donors (Figure 5). Under these conditions, free phosphorylcholine (Ch-P) and CDP-choline (CDP-Ch) did not support SMS1- or SMS2-mediated SM synthesis (Figure 6A). PC, on the other hand, was efficiently recognised as  
20 substrate. SM itself was also used as a donor of the phosphorylcholine group. Lyso-PC was a very poor substrate. These results suggest that human SMS1 and SMS2 are transferases that require two fatty chains on the choline-P donor molecule in order to be recognised efficiently as a substrate.

### 25 Example 4

The reaction of human SMS1 or SMS2 with non-choline phospholipids

We next investigated the possibility that human SMS1 or SMS2 would use non-choline phospholipids as substrates. None of the phospholipids tested  
30 (PE, PI, PS, PA, PG) other than PC supported SM formation (Figure 6A).

Moreover, when extracts of cells expressing SMS2 were incubated with PC containing [<sup>3</sup>H]-choline, the formation of radiolabelled NBD-SM was observed (Figure 6B). Hence, it appears that SMS1 and SMS2 directly and specifically recognise the choline head group on their substrates.

5

### Example 5

#### The reaction of sphingomyelin and diacyl glycerol

Previous work suggested that mammalian SM synthase is also capable of catalysing the reverse reaction, namely the formation of PC from SM and DAG (Marggraf and Kanfer, 1984; van Helvoort et al., 1994).

To investigate whether this was also the case for human SMS1 and SMS2, extracts of cells expressing these proteins were incubated with NBD-DAG and the formation of NBD-PC monitored by TLC. Addition of SM induced NBD-PC formation in extracts of SMS1- or SMS2-expressing cells, but not in control cell extracts (Figure 7). Strikingly, PC itself proved more efficient than SM in stimulating SMS1- or SMS2-dependent NBD-PC formation. In contrast, addition of non-choline phospholipids (e.g. PI) had no effect. These data suggest that human SMS1 and SMS2, rather than functioning strictly as SM synthases, are transferases capable of using PC and SM as phosphocholine donors to produce PC or SM, dependent on the relative concentrations of DAG and ceramide as phosphocholine acceptors, respectively.

Mammalian SM synthase was found to be sensitive to the bacterial PC-phospholipase C inhibitor, D609 (Luberto and Hannun, 1998). In lysates of SMS1- or SMS2-expressing yeast cells, D609 inhibited SM synthesis in a dose-dependent manner. The extent of inhibition observed for SMS1-mediated SM synthesis (50% at 100 g/ml D609) was comparable to that reported for mammalian SM synthase (Luberto and Hannun, 1998). SMS2 proved two-fold less sensitive to the drug.

Hence, human SMS1, SMS2 and the mammalian SM synthase activity described in the literature share many enzymatic characteristics.

### Example 6

#### 5                    Structure and expression of human SMS1 and SMS2

Figure 8A shows a sequence alignment of human SMS1, SMS2 and SMSr. Hydrophobicity analysis predicted six membrane-spanning alpha helices connected by hydrophilic regions that would form extramembrane  
10 loops. A comparative analysis with SMS and SMSr sequences from mice, *C. elegans*, *P. falciparum* and *Drosophila* revealed that the number and spacing properties of transmembrane helices are well conserved. In addition, SMS proteins contain four highly conserved sequence motifs, designated D1, D2, D3 and D4 (Figure 8A and B, residues highlighted in blue). Motifs D3 (C-G-D-X<sub>3</sub>-  
15 S-G-H-T) and D4 (H-Y-T-X-D-V-X<sub>3</sub>-Y-X<sub>6</sub>-F-X<sub>2</sub>-Y-H) are similar to the C2 and C3 motifs in LPPs (shared residues highlighted in red) and include the histidine and aspartate residues (underlined) that form a catalytic triad mediating the nucleophilic attack on the lipid phosphate ester bond Neuwald (1997).

20                    As in LPPs, these residues are juxtaposed to transmembrane segments 4 and 6 of the SMS proteins and consequently would be oriented toward the same side of the membrane. This would suggest that motifs D3 and D4 are part of the catalytic site responsible for liberating cholinephosphate from PC during SM synthesis. Motifs D1 (P-L-P-D) and D2 (R-R-X<sub>8</sub>-Y-X<sub>2</sub>-R-X<sub>6</sub>-T), on the  
25 other hand, appear entirely unique to SMS proteins and are located in the first extramembrane loop and third transmembrane helix, respectively (Figure 8A). The SMSr proteins found in humans, mouse, *Drosophila* and *C. elegans* each contain exact copies of the D1 and D3 motifs, yet exhibit one or more conserved

amino acid substitutions in motifs D2 and D4 (Figure 8A and B, residues highlighted in white on grey).

Previous work showed that some members of the mammalian LPP superfamily are expressed in only a limited set of tissues (Waggoner et al., 1999, Brauer et al, 1997).

Since differences in tissue distribution would provide a possible explanation for the existence of two different SM synthase isoforms in mammals, we investigated the expression profiles of human SMS1 and SMS2. As shown in Figure 10, Northern blot analysis detected the presence of a low abundant 3.8 kb transcript for SMS1 in human brain, heart, kidney, liver, muscle and stomach. A major 1.9 kb transcript for SMS2 was expressed to a similar level in all of the above human tissues. These results suggest that human SMS1 and SMS2 are encoded by ubiquitously expressed genes.

15

### Example 7

#### Subcellular localisation and membrane topology

SM synthesis occurs in the Golgi complex as well as at the plasma membrane of mammalian cells. Endosomes have been put forward as another major site of SM synthesis (Kallen et al., 1994) but this has been disputed (van Helvoort et al., 1994).

It is not known whether SM synthase activity detected at these locations is due to the presence of more than one iso-enzyme in the cell. This led us to examine the subcellular distribution of V5-tagged versions of human SMS1 and SMS2 in transfected HeLa cells using immunofluorescence microscopy. As shown in Figure 11A, SMS1 was concentrated in the perinuclear region where it displayed extensive colocalisation with sialyltransferase, a marker of trans Golgi cisternae. This colocalisation was also observed in cells treated with nocodazole, a drug causing fragmentation of the Golgi by disrupting the microtubular network, hence confirming the association of SMS1 with the

Golgi apparatus. SMS2 displayed a different localisation pattern and was primarily concentrated at the plasma membrane (Figure 11B). A portion of SMS2 was also found in the perinuclear region where it colocalised with sialyltransferase (see Figure 12). This Golgi-associated pool of SMS2 unlikely  
5 represents newly synthesized material en route to the cell surface, since it was also observed in cells after a 4-hour chase with cycloheximide. There was no substantial colocalisation of SMS2 with markers of the endosomal/lysosomal system (e.g. EEA1, CD63, internalised transferrin; data not shown). Whether SMS2 cycles between the Golgi and the plasma membrane, and thereby passes  
10 through endosomes remains to be established.

Since SM synthesis takes place in the exoplasmic leaflet of the Golgi and the plasma membrane (Futerman et al., 1990; van Helvoort et al., 1994), the putative catalytic residues in motifs D3 and D4 of SMS1 and SMS2 would be oriented toward the Golgi lumen and cell surface, respectively, whereas their  
15 termini would be located on the opposite, cytosolic side of the membrane (Figure 13A). To test this prediction, we investigated the sidedness of the V5-tagged COOH termini of human SMS1 and SMS2 by protease protection analysis. When HeLa cells transfected with the SMS2-V5associated type I membrane protein, p24 construct were trypsinised, the V5 tag remained  
20 intact, unless cells were lysed or treated with detergent prior to incubation with the protease (Figure 13B). Trypsinisation of lysates from SMS1-V5 expressing cells in the absence of detergent resulted in a complete removal of the V5 tag. Under these conditions, the Golgi-associated type I membrane protein, p24 (Gommel et al., 1999).  
25 was largely protected. Collectively, these results indicate that the COOH termini of SMS1 and SMS2 are cytosolic. Consequently, the putative active site residues in these proteins would be positioned on the exoplasmic leaflet (Figure 13A), hence where SM synthesis was found to occur. Together, these findings demonstrate yet another level of similarity between SMS1, SMS2 and  
30 the SM synthase activity previously described in mammalian cells.

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**FIG. 9**

## Sequence listings

Sequence listings 1 - 11: conserved sequence motifs of phosphotransferases as indicated in figure 8 B;

sequence listing 1: partial polypeptide sequences of *hu* SMS1  
sequence listing 2: partial polypeptide sequences of *hu* SMS2  
sequence listing 3: partial polypeptide sequences of *ce* SMS1  
sequence listing 4: partial polypeptide sequences of *ce* SMS2  
sequence listing 5: partial polypeptide sequences of *ce* SMS3  
sequence listing 6: partial polypeptide sequences of *pf* SMS1  
sequence listing 7: partial polypeptide sequences of *pf* SMS2  
sequence listing 8: partial polypeptide sequences of *hu* SMSr  
sequence listing 9: partial polypeptide sequences of *dm* SMSr  
sequence listing 10: partial polypeptide sequences of *ce* SMSr  
sequence listing 11: partial polypeptide sequences of *ce* SMSdr

Sequence listing 12: *hu*SMS1

MKEVVYWSPKKVADWLLNAMPEYCEPLEHFTGQDLINLTQEDFKKPPLCRVSSDN  
GQRLLDMIETLKMEHHLEAHKNGHANGHLNIGVDIPTPDGSFSIKIKPNGMPNGYRK  
EMIKIPPELERSQYPMEWGKTFLAFLYALSCFVLTTVMISVVHERVPPKEVQPPLPD  
TFFDHFNRVQWAFSICEINGMILVGLWLIQWLLKYSIISRRFFCIVGTLYLYRCITM  
YVTTLPVPGMHFNCSPKLFGDWEAQLRRIMKLIAGGGLSITGSHNMCGDYLYSGHTV  
MLTLTYLFIKEYSPRRLWWYHWICWLLSVVGIFCILLAHDHYTVDVVVAYYTTTRLF  
WWYHTMANQQVLKEASQMNLLARVWWYRPFQYFEKNVQGIVPRSYHWPFPPWPV  
VHLSRQVKYSRLVNDT

Fig 9 (cont.)

Sequence listing 13: *huSMS2*

MDIETAKLEEHLNQSDPTNTYARPAEPVEEENKNGNGKPKSLSSGLRKGTKKYPD  
YIQIAMPTESRNKFPLEWWKTGIAFIYAVFNVLTTVMITVVHERVPPKELSPPLPDKF  
FDYIDRVKWAHSVSEINGHILVGLWITQWLFLRYKSIVGRRFCFIIGTLYLYRCITMYVT  
TLPVPGMHFQCAPKLNGDSQAKVQRILRLISGGGLSITGSHILCGDFLFSGHTVTLTLT  
YLFIKEYSPRHFWWYHLICWLLSAAGUCILVAHEHYTIDVHA YYITTRLFWWYHSMA  
NEKNLKVSSQTNFLSRAWWFPIFYFFEKNVQGSIPCCFSWPLSWPPGCFKSSCKKYSR  
VQKIGEDNEKST

Sequence listing 14: *ceSMS1*

MKMSWNHQYTNYGSIADDNGDEEKAENSEGAAAEKVEKQHDDDGVVVHEETDGV  
ASSRSSHHDKQKPGETKKSGDGKMDDDDIITARSSSRICGSAASSSDSETADDAPL  
LPDEGPSHAVRLEMPGDKPASPHDRFPKTPKTLVAFMLLVVAAAGNTITLSWIHER  
YPLTPPLPDIVFELIPKPWGLRLCENLMIGSFVSLVLILFHRHRWTVLRRLCFIGSILYG  
MRCITMMVTPVPKADEDFECSPRFGENATFSLIVMRGVWSMFGLGLNLFDNQKVVL  
CGDYIYSGHTLVLVVSALFIGEYSPRRFYILHWLSWLVCSVGVI FLVLSHGHTIDVIL  
SYFACTRVFWAYHTQAAHPSIRLSVQNHQAKEFWFLLRWFECDIRRPVPRRFDCPIS  
YSQTPVDYSFLPWFSFFFQSNPMMFPGAFTFYHTQNHSKSTMSLPK

Sequence listing 15: *ceSMS2*

MTNSSEFTDVLQSRDPCVSNQIVINIDPIDPEPTPIRKEFTCEDTFHHEHHGNSEGFKTL  
TAFCLCLMSAFLNFFLLTVIHDVVPRQPLPDLTFMIPQQRWAWSGDVLSTVSSVVA  
FTIIFLHHQRWTVLRRTFLLGAIMYGLRAVILGVTFLLPSFHNREICQPQVNRTAMYG  
MEIATRFILTYVITLGLTSGQDKILCGDLMFSGHTVVLTIMYFVQLQYTPRGLVILRYLA  
APITFLGIAALVVSGGHYTMDVLIAYWLTSHVFWSYHQIFEMRKDDRPQAPLSRLW  
WFWLCYWFESDVADGKLVNKWNPLEGPQRMHTIMNRINYKLQ

Fig 9. (Cont.)

Sequence listing 16: *ceSMS3*

MGSVSKTVISARGASPDDEQNGTKNGISNGSEWAKCIFLFFFLFIAGMSNWAVLAYT  
HDYVPRESLPDIVFSLVSEQRWASSLGDFCVAlCIVMLGALLVIHQHRGTILKR VVFC  
AGTLYAMRSVTLAATQLPSGYTDNQGRCDQVESEAGVFFGRLFEQTIRIGFQSKDQ  
MLCGDLLFSGHTLVMVTCSLAVAYYLPKSIKPLQWVSHVACLIGMICMTISRTHYTD  
VVIAYWLSNMVFRMYHAYCEVDMCMERRKSILYSWWPCRIVDWLEQDIVPGRLEN  
RCQLPWRRSTPRGQERGGASAESSDSSVTMCDNITTSHHQKHVSISSSSTYPLPC

Sequence listing 17: *pfSMS1*

MIEGNNANTTHQNTTNNKIEKNFIKEKDINNDTIKILIEHNNSFNDESTNYITTFNDIK  
DKHYNHKDSYSDNKGKIKTLRSNFSDIQDEVITNIGHYFEGQENIKKSVEIKNCPYKK  
KMLKIFLRRLVCATIGIGICIQCYPILSDTYKYTGDEPLKDRLEIFKEIPAFMNTPFV  
NGSIMFFLAITLLRFGFCPLLSITILIRIILMLSFTYCI RSFFIYVTTLPCPIPTCQPLKHKT  
LVENLYTFYLIITAQVYECTDLVISGHTAFTTLLTFFWFFYERNIYVKTTIFLYSIYIYIIII  
SRFHYTVDVLMGYVFGGSVFLFYHYLVDVAARRYALNTSVFPQTYGFSGSFTDRFQ  
VFQYFIRAITYLEALDHRMNFALS YDKEWNCFCSCVPVNKNSLLIKKKNVRNEEYYD  
FSDHFYHSYAGNGTYNLSTIRNIIKEFKRFFGMNKK

Sequence listing 18: *pfSMS2*

MNKYEYFYSPIQLLHLILLRPILYENYTYDENDKSSIGTDMYEINMNRKMSNISISRN  
STINEEELSEYRLCKILLIKLMFALLFLIALIIQGFFMIYSDSYKSNTOPLSDRIHDLF  
GNPPKWISYKLSNTLIAILTL SFLKIILFNSIYLSIAIICRFLYIVGSFYIIRGLLIYVTSLPAT  
LETCLPLESGNFLFNLLQIIKINTNLVYVCADLIVSGHSFSTTIFLMFSFYINNVIKFIIF  
TFSCFIYAIHIGFIHYTSDVLLGIIFGVFMFSFYHIMLDISSQYYTFNKLFEIKIISNNKNIHA  
KPFFLRFFVARIFFKIIPYLEGLNYTLDYAINKNNDLSTFCNCDHDNNKIPLFSFYKPITE  
DKIINYS D HLYHSYAGDGTINFLFWKFLKTIKKGLHK

Fig 9. (cont.)

Sequence listing 19: *huSMSr*

MAGPNQLCIRRWTTKHVAVWLKDEGFFEYVDILCNKHRLDGITLLTLTEYDLRSPPL  
 EIKVLGDIKRLMLSVRKLQKIHDVLEEMGYNSDSPMGSMTPFISALQSTDWLCNGEL  
 SHDCDGPITDLNSDQYQYMNGKNKHSVRRLDPEYWKTILSCIYVFIVFGFTSFIMVTV  
 HERVPDMQTYPPPLDIFLDSVPRIPWAFAMTEVCGMILCYIWLLVLLLHKHRSILLRRL  
 CSLMGTVFLLRCFTMFVTSLSVPGQHLQCTGKIYGSVWEKLHRAFAIWSGFGMTLTG  
 VHTCGDYMFSGHTVVLTMLNFFVTEYTPRSWNFLHTLSWVLNLFGIFFILAAHEHYSI  
 DVFIAFYITTRLFLYYHTLANTRAYQQSRRARIWFPMFSFFECNVNGTVPNEYCWPFSS  
 KPAMKRLIG

Sequence listing 20: *dmSMSr*

MCDGEIGDPVTQPRSEGGGLVTMDQETRTHYLDAATDKHLTNGSPDPEPVDPLLVA  
 QWSIENVTSWATCMEHFSRTLDDCLRQEAIIDGEVLLSLTEEDVRDMRYKLGKLTFG  
 ELKKFWIAVLKLQLLVKNSSAESVILGIECHGNGNSVYMPLASTGCGPPSSSTCPCPQ  
 AECPSYVSDCDTYLRMGGRYPPEYFKTAMSLGYSFVVTWITSLTMVIVHERVPDM  
 KRYPPPLDIFLDNVPHIPWAFNMCEITGSLLFTTWVVVLTFFHKYRLVLLRRFFALAGTV  
 FLLRCVTMLITSLSVPGTHLQCSQKDFAIIDPNVDMVGALIIRMSRAYRIWSGLGMSI  
 QGVRTCGDYMFSGHTVALTLLNFFITEYTPRNLYFLHTLTWLLNMFGIFFILAAHEHY  
 SIDVFVAFYITSRLFLYYHTLANNRALMQSDSKRTRVWPFMFSSYFESSVDGMVPNEY  
 DTLGSLIDGIEQIFKAKDQLAISVKRCWLDAPLSGNSSAHMFGGESEQCLRNGTASA  
 AFFSPHQSLIGGLGGQRSQTHLNSAQSGSSTPTPATSAPTKSLPTQKKTFRDASVDPFS  
 RTTFAAVQQAENVKDSPMKEKKHL

Sequence listing 21: *ceSMSr*

MLDNRPADPNEWRCEDVGNWLKKIGMAKYADLIAMKHKVDGKCLLALTDIDLK  
 DPPVSINCLGDIKKILFAIEFLSQKVVEIGNSGVHHRSTPNNGPSLKNKDGLLVEYN  
 EQNHLSISGEDVYTTTTRRAEIVEDEETLLDTLAKSSDGTSTVQLISREEIRQVERPDY  
 FKSVAKLLIAFAYSSLSFLMTSFVMVLVHDRVDPDTKTYPPPLDIVLDNVPHIPWAFDM

Fig 9 (Cont.)

57

CETIGLVLA VVWFTVLFFHNQRVIVARRMFSL LGTVFLLRCFTMLITSLSVPGIHLQCE  
ARPNTTMQEKLHKAFHIWSNLGMSLHGVRSCGDYMFSGHTTVITMISHFITEYTPAD  
WTGLHTFTWVLNCF AFLILAAHEHYSIDVFIAFYISSRMFLYYHAYAYNHAGITATD  
YRMRTWFPLGWFFEYGSQGKVENEFSLPINIRPRRVFFAKSEEPKITPKSDSSRKRSS  
VVA AKQNGNSKNHTKKHN

Sequence listing 22: *ceSMSdr*

MCKSCENYGKFFFAMLKICIDNSSNFAMKKDAENENLLHAYEHDEQSSKYFIKPDSR  
YSIDDDISVKIDGKQLEVRKWPTLLATAMVGVGWLSNEVALAWVHERVPDDYHPLP  
DLFFSHFPEVKERLKIFGILGKFEKSEKKTSEFSSPIRGAIRIAEYIMMILLISALLVMFTH  
QHRWIVIRRVFFCIAMAYSFRALCVTIFQVPVPSINTYCAPKSNSSLELVAGR VVKMF  
WSAGIEQLRPRELCGDLIVSGHTLTIFTAFLVKTYAPQRLQPLSHIYHVLAFTALFSIL  
LARKHYMIDIVLGYTVSTRIFMEYHALAASYHNRTFETNPLAWSFWSFFIPIFECDAPA  
NMHNHLLLYNRSTSSKNVSTLKKSRRSFE

## Claims

- 5 1. An isolated polypeptide comprising one or more of the amino acid motifs selected from the group consisting of a sequence with at least 80% identity to any of
- 10 (a) P-L-X-D-X(35,75)-R-R-X(8)-[YF]-X(2)-R-X(6)-T
- (b) C-X-D-X(3)-S-G-H-T
- (c) H-Y-[TS]-X-D-[VI]-X(3)-[FYI]-X(6)-F-X(2)-Y-H.
- 15 2. A polypeptide according to claim 1 that is derived from the group consisting of *Animalia*, *Alveolata* and *Kinetoplastida*.
3. A polypeptide according to claim 1 or 2 selected from the group comprising any of the SEQ ID No. 1 - 11 or at least 70% similarity thereto.
- 20 4. A polypeptide according to claim 1 or 2 comprising an amino acid sequence with at least 70 % similarity to any of the SEQ ID No. 12 - 22.
- 25 5. A polypeptide according to any one of the previous claims comprising an amino acid sequence with at least 20 % identity to any of the SEQ ID No. 12 - 22.
- 30 6. A polypeptide according to claim 5 comprising an amino acid sequence with at least 30 % identity to SEQ ID No. 12.

7. A polypeptide according to claim 5 comprising an amino acid sequence with at least 40 % identity to any of the SEQ ID No. 19 - 21.
- 5
8. A polypeptide according to any one of the claims 1 - 3 comprising an amino acid sequence with at least 22 % identity to SEQ ID No. 22.
- 10 9. A polypeptide according to any one of the claims 1 - 6 with sphingomyelin synthase activity.
- 15 10. A polypeptide according to any one of the claims 1 - 5 and 7 with ethanolamine phosphorylceramide synthase activity.
11. A polypeptide according to any one of the claims 1 - 5 and 8 with one or more of the activities selected from the group consisting of phosphatidylcholine:glycoprotein cholinephosphotransferase and phosphatidylcholine:glycolipid cholinephosphotransferase.
- 20
12. A nucleotide sequence selected from the group consisting of a nucleotide sequence coding for any of the amino acid sequences as described in claim 9 and an anti sense nucleotide sequence that is complementary thereto.
- 25
13. A nucleotide sequence selected from the group consisting of a nucleotide sequence coding for any of the amino acid sequences as described in claim 10 and an anti sense nucleotide sequence that is complementary thereto.
- 30 14. A nucleotide sequence selected from the group consisting of a nucleotide



sequence coding for any of the amino acid sequences as described in claim 11 and an anti sense nucleotide sequence that is complementary thereto.

15. A plasmid comprising any of the nucleotide sequences described in any  
5 of the  
claims 12, 13 or 14.

16. A vector comprising any of the nucleotide sequences described in any of  
the claims 12, 13 or 14.

10 17. A (micro)organism or cell line in which any of the nucleotide sequences  
described in claim 12 was introduced.

18. A (micro)organism or cell line in which any of the nucleotide sequences  
15 described in claim 13 was introduced.

19. A (micro)organism or cell line in which any of the nucleotide sequences  
described in claim 14 was introduced.

20 20. A process for producing sphingomyelin synthase comprising the  
expression of  
any one of the nucleotide sequences described in claim 12 in a (micro)organism  
or cell line of claim 17 and the isolation of sphingomyelin synthase.

25 21. A process for producing sphingomyelin comprising the expression of the  
nucleotide sequences described in claim 12 in a (micro)organism or cell of claim  
17 and the isolation of sphingomyelin.

22. Use of one of more of the nucleotide sequences of claim 12 to influence  
30 the reaction



*in vivo or in vitro.*

5

23. Use of one or more of the nucleotide sequences of claim 12 to identify or develop compounds influencing the reaction

10



*in vivo or in vitro.*

15

24. A process for producing ethanolamine phosphorylceramide synthase comprising the expression of any one of the nucleotide sequences described in claim 13 in a (micro)organism or cell line of claim 18 and the isolation of ethanolamine phosphorylceramide synthase.

20

25. A process for producing ethanolamine phosphorylceramide comprising the

expression of the nucleotide sequences described in claim 13 in a (micro)organism or cell line of claim 18 and the isolation of ethanolamine

25

phosphorylceramide.

26. Use of any one of the nucleotide sequences of claim 13 to influence the reaction

5 CER + PE  $\leftrightarrow$  EPC + DAG

*in vivo* or *in vitro*.

27. Use of any one of the nucleotide sequences of claim 13 to identify or  
10 develop compounds influencing the reaction

CER + PE  $\leftrightarrow$  EPC + DAG

15 *in vivo* or *in vitro*.

28. The application of the compounds of claim 23 or 27 in medical use.

29. The application of the compounds of claim 28 for the manufacture  
20 of medicaments treating a disease selected from the group consisting of cancer, metabolic diseases and diseases caused by parasites.

30. A process for producing phosphatidyl:glycoprotein  
cholinephosphotransferase or phosphatidyl:glycolipid  
25 cholinephosphotransferase comprising the expression of any one of the corresponding nucleotide sequences described in claim 14 in a (micro)organism or a cell line of claim 19 and the isolation of phosphatidyl:glycoprotein  
cholinephosphotransferase or phosphatidyl:glycolipid  
cholinephosphotransferase.

30

31. A process for producing phosphorylcholine-substituted glycoprotein or

phosphorylcholine-substituted glycolipid comprising the expression of the corresponding nucleotide sequences described in claim 14 in a (micro)organism or cell of claim 19 and the isolation of phosphorylcholine-substituted glycoprotein or

5 phosphorylcholine-substituted glycolipid .

32. Use of one of more of the nucleotide sequences of claim 14 to influence the reaction

10 glyco lipid/protein + PC  $\leftrightarrow$  PC-substituted glyco lipid/protein + DAG

*in vivo* or *in vitro*.

33. Use of one of more of the nucleotide sequences of claim 14 to identify or  
15 develop compounds influencing the reaction

glyco lipid/protein + PC  $\leftrightarrow$  PC-substituted glyco lipid/protein + DAG

*in vivo* or *in vitro*.

20

34. The application of the compounds of claim 33 in medical use.

35. The application of the compounds of claim 34 for the manufacture of a medicament treating a disease caused by parasitic nematodes.

25

36. A process to isolate candidates for functional genes of a previously unidentified enzyme with known activity from a huge database by combining at least four characteristics based on data from bio-informatics and from biochemistry, viz.

- presence of a sequence motif shared with previously identified enzymes having a related function
- biochemical function of the gene should be unknown until now
- no structural homologues in an organism that does not contain the enzyme
- 5 – ability to mediate a reaction catalysed by the unidentified enzyme upon its heterologous expression in an organism or cell lacking said enzyme activity.

37. A process to isolate candidates for functional genes according to claim 36 characterized by also considering the presence or non-presence of  
10 transmembrane domains depending on the working mechanism of the enzyme in relation to the membrane.

38. A method for determining whether a compound is capable of modulating an enzymatic activity displayed by a cell, said activity comprising  
15 an activity of an enzyme of the group of enzymes identified as sphingomyelin synthases, ethanolamine phosphorylceramide synthases, phosphatidylcholine:glycoprotein cholinephosphotransferase and phosphatidylcholine:glycolipid cholinephosphotransferase, said method comprising providing said cell with a nucleic acid encoding a polypeptide  
20 according to any one of claims 1-11, contacting said cell with said compound and determining whether said enzymatic activity is modulated.

39. A method according to claim 38 wherein said cell is deficient in sphingomyelin synthase activity.  
25

40. A method according to claim 38 or claim 39, wherein said cell is a cell of a eukaryotic micro-organism.

41. A method according to claim 40, wherein said cell is yeast cell.  
30

42. A method according to any one of claims 38-41, wherein said polypeptide comprises a sequence as depicted in figure 8 or a functional part, derivative and/or homologue thereof.
- 5 43. A method according to claim 42, wherein said polypeptide comprises a sequence as depicted in figure 8A or a functional part, derivative and/or homologue thereof.
44. A method according to any one of claims 38-43, wherein said  
10 polypeptide is derived from a plasmodium.
45. A method according to claim 44, wherein said plasmodium sequence is a sequence as depicted in figure 8B or a functional part, derivative and/or homologue thereof.
- 15 46. A method according to any one of claims 38-45, wherein said compound comprises RNA.
47. Use of a nucleic acid encoding a polypeptide according to any one of  
20 claims 1-11, as a probe.
48. Use of an oligonucleotide specific for a nucleic acid sequence encoding a polypeptide as depicted in figure 8 or a functional part, derivative and/or homologue thereof, for detecting said sequence.
- 25 49. Use according to claim 47 or claim 48, for assessing whether a cell comprises sphingomyelin synthase activity.
50. Use of an inhibitor of a sphingomyelin synthase according to any one  
30 of claims 1-11, as a cell death promoter.

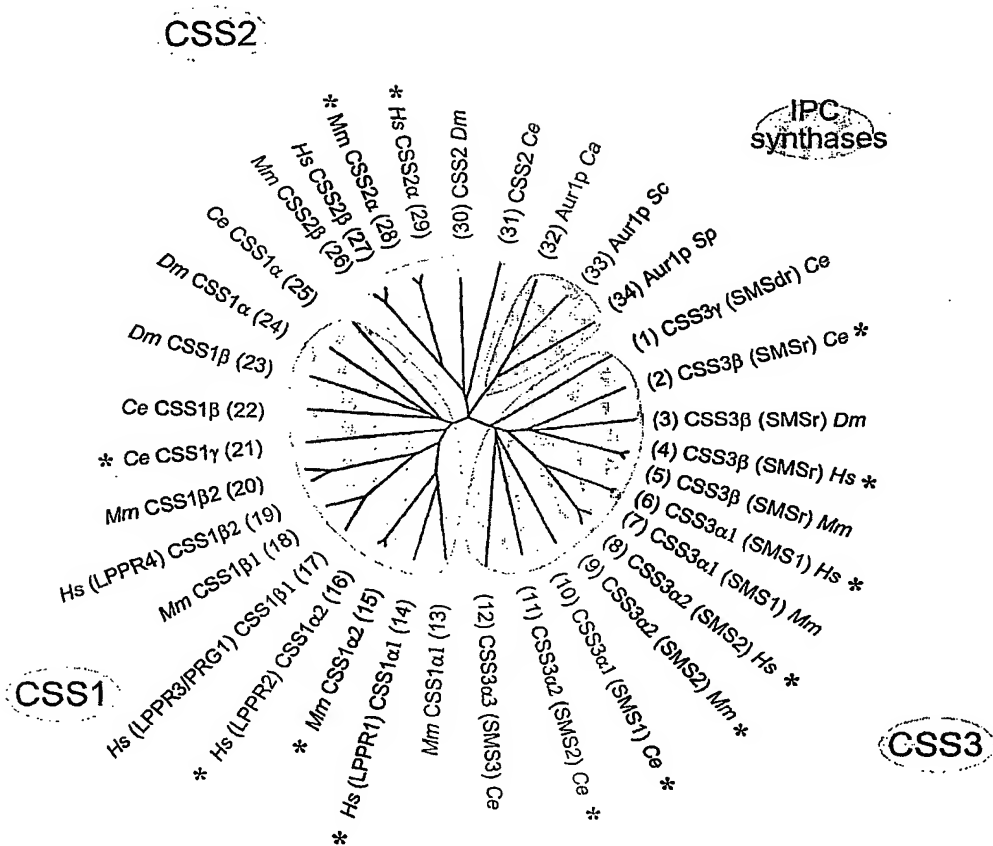
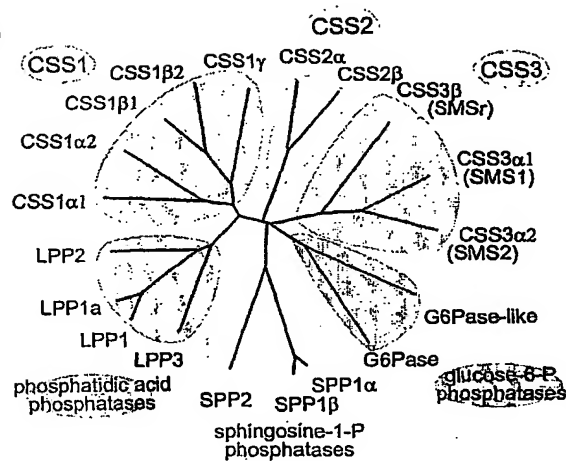
51. Use according to claim 50, wherein said cell is a cell of a parasite.
52. Use according to claim 50, wherein said cell is a human cell,  
5 preferably a tumor cell.
53. Use of a nucleic acid according to any one of claims 12-14, preferably  
comprising a nucleic acid sequence encoding a polypeptide as depicted in figure  
8 or a functional part, derivative and/or homologue thereof for enhancing cell  
10 survival and/or cell growth.
54. A method for at least in part improving the yield of an secretion  
product of a cell comprising providing said cell with a polypeptide according to  
any one of claims 1-11, or a nucleic acid according to any one of claims 12-14,  
15 preferably comprising a nucleic acid sequence encoding a polypeptide as  
depicted in figure 8 or a functional part, derivative and/or homologue thereof.
55. A method according to claim 54, wherein said cell is a cell of a  
eukaryotic micro-organism.  
20
56. A method according to any one of claims 38-46, further comprising  
providing said cell or a fraction thereof with a labelled substrate for said  
sphingomyelin synthase.
- 25 57. A method according to claim 56, further comprising harvesting  
sphingolipid from said cell or said fraction and detecting labelled sphingolipid.
58. A method according to claim 56 or 57, further comprising detecting  
said labelled sphingolipid using (thin layer) chromatography or mass  
30 spectrometry.

59. A method for targeting a first polypeptide according to any one of claims 1-11 to a different cellular compartment comprising providing a cytosolic part of said first polypeptide with a cellular compartment localization signal of a cytosolic part of a second polypeptide according to any one of claims 1-11, wherein said first and said second polypeptide, when unmodified, reside in different cellular compartments.
60. A method according to claim 59, wherein said cytosolic part of said first polypeptide comprises the C-terminal end of said polypeptide.
61. A method according to claim 59 or claim 60, wherein said cytosolic part of said second polypeptide comprises the C-terminal end of said polypeptide.
62. A method according to any one of claims 59-61, wherein said cellular compartments comprises the plasma membrane, the endosomal compartment, the Golgi, the endoplasmatic reticulum or a combination thereof.
63. A method according to any one of claims 59-62, wherein said cellular compartment localization signal of said second polypeptide replace the C-terminal cytosolic part of said first polypeptide.



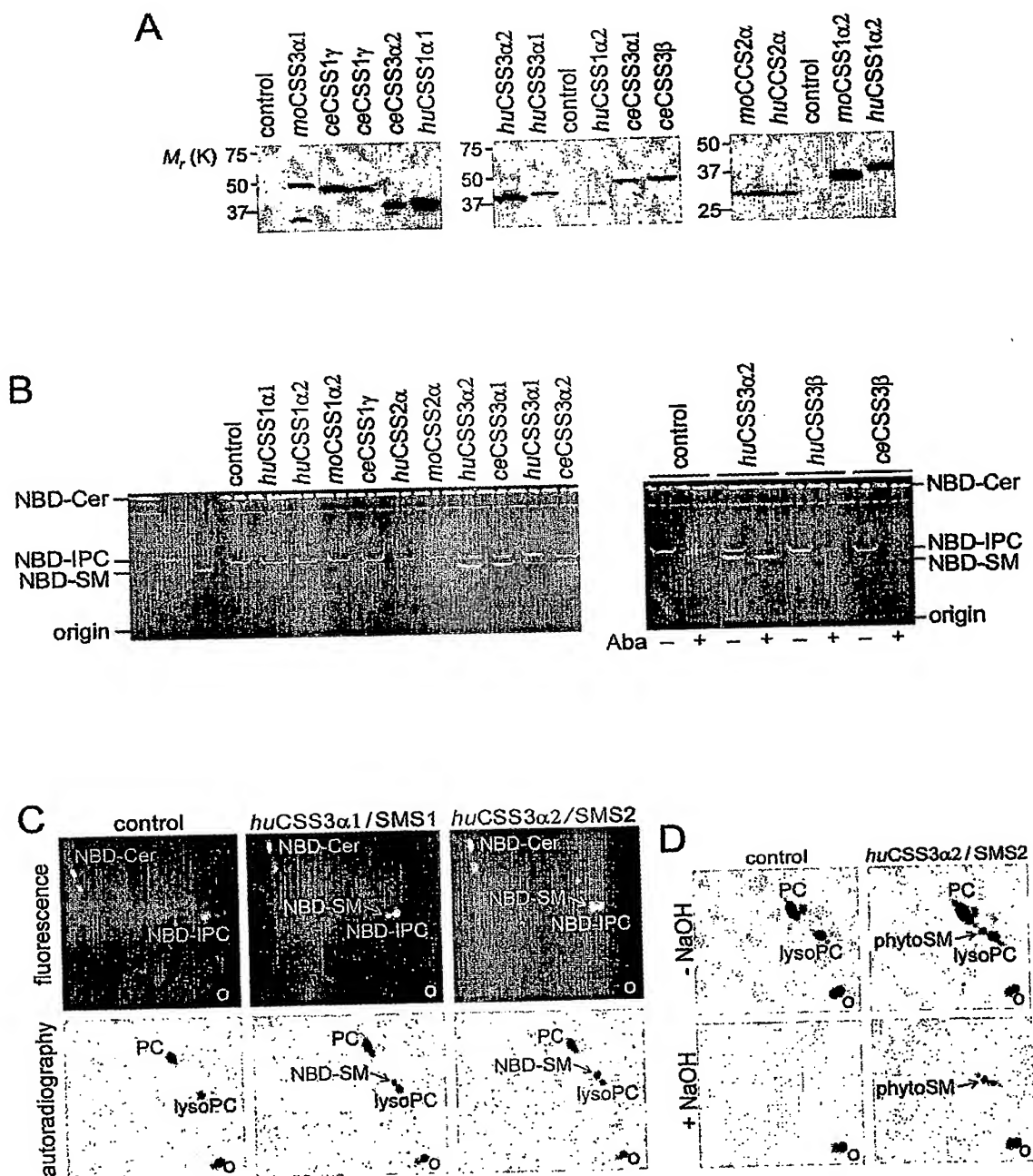
## B

31 candidate SM synthase sequences  
*human* (9), *mouse* (9), *worm* (9), *fly* (4)



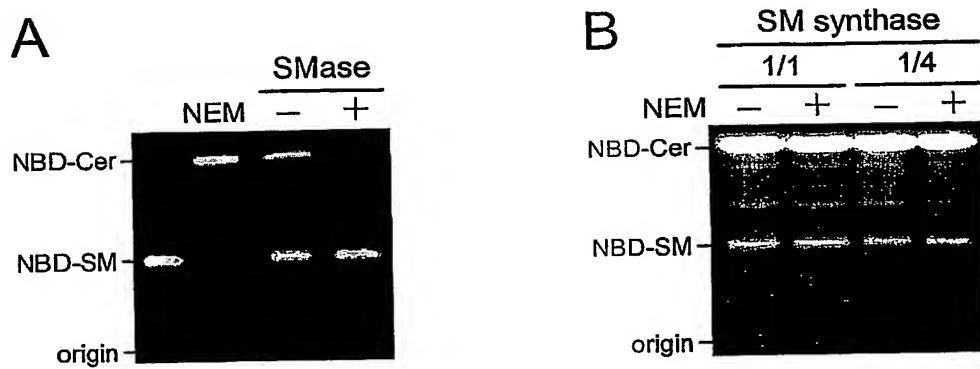
2/13

Fig. 2



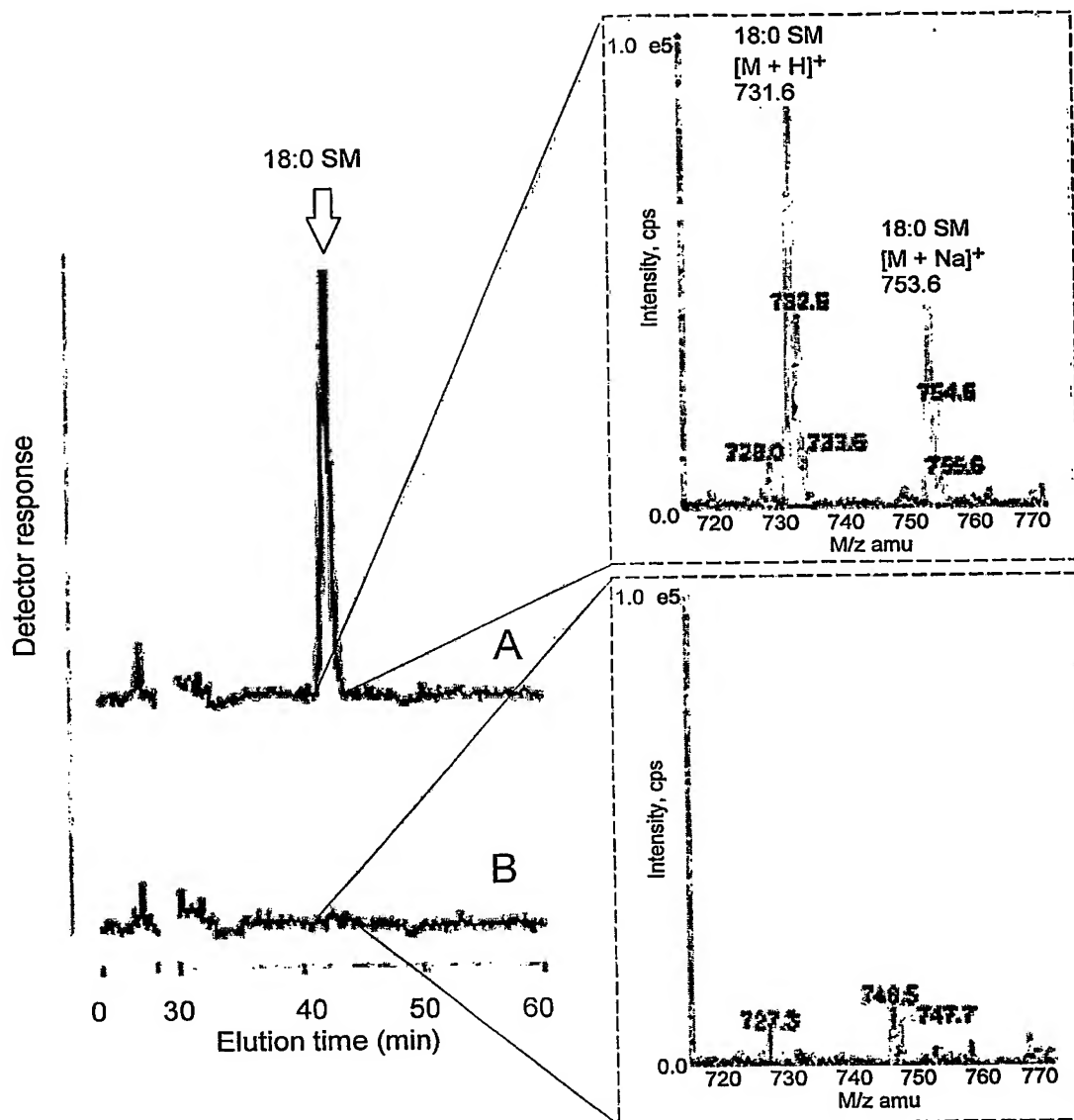
3/13

Fig. 3



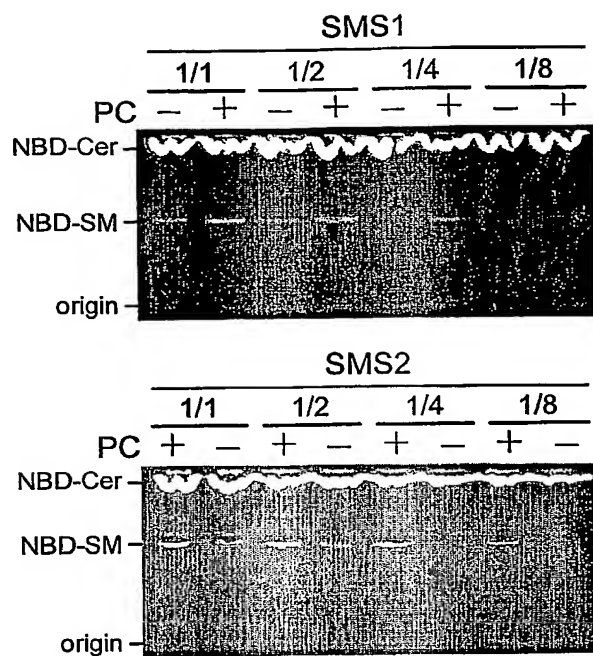
4/13

Fig. 4



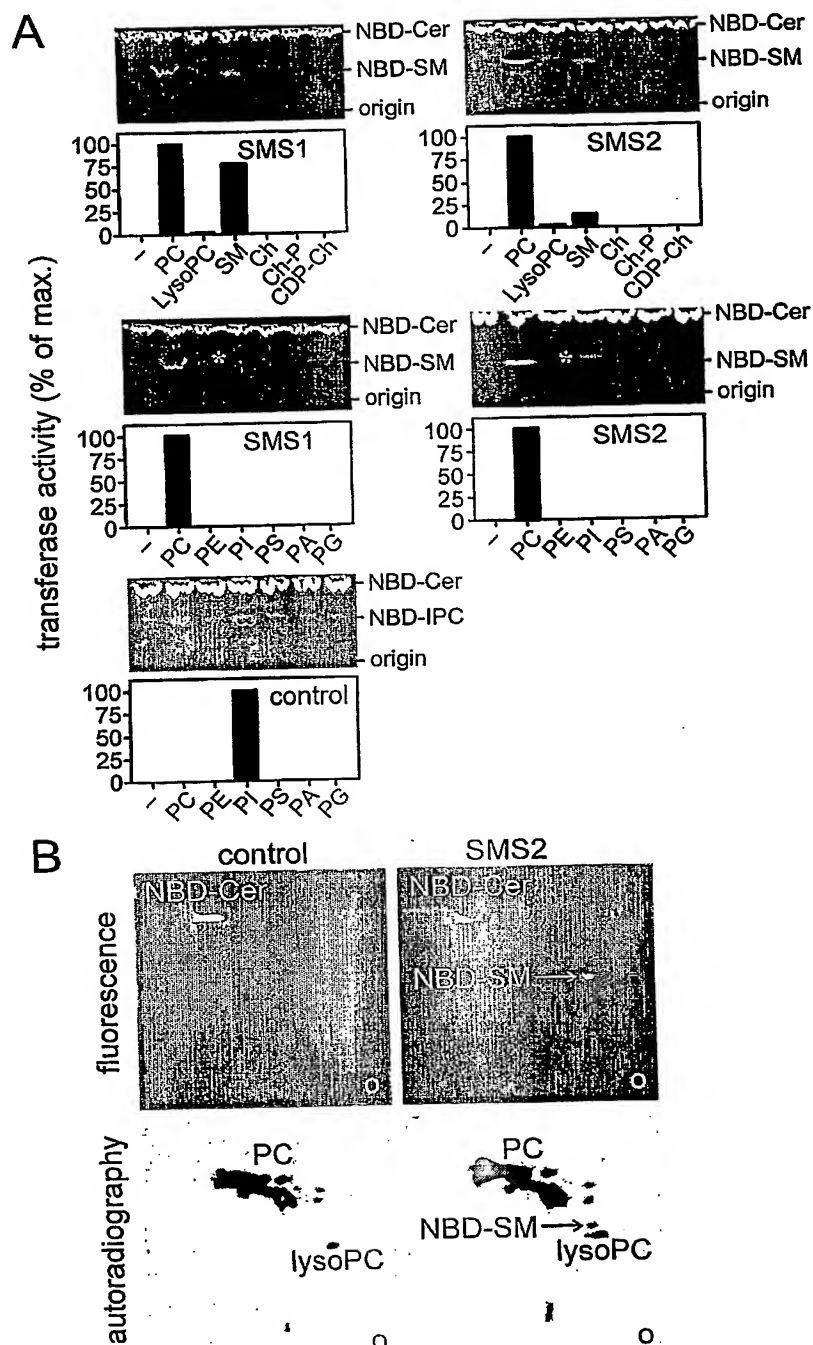
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Fig. 5



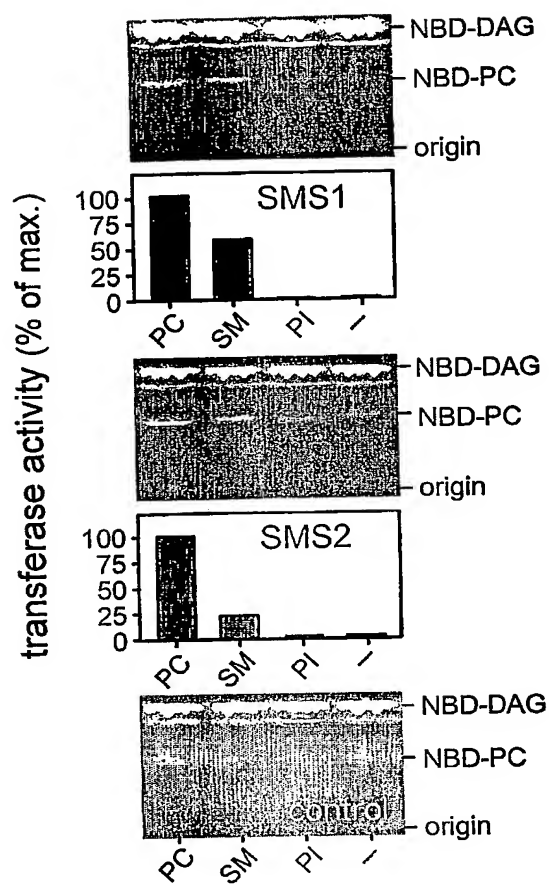
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Fig. 6



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Fig. 7



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Fig. 8

A

Hs SMS1 1 MKEVYVWSPKKVADWLENAMPEYCEPLE---HFTGQDLINLTQEDFKKPPLCRVSSDNIG  
 Hs SMS2 1 MAGPNQLCIRRWTTKHVAVWLKDEGFEEYVDILCNKHRLDGI TLLTLEVDLRSPPLEIKVLGD I  
 Hs SMSr 1

Hs SMS1 58 QRLDM IETLKMEHH--LEAH---KNGHANGLN--IGVDIPTPDGSFSIKIKPNG-MPNGYRK-  
 Hs SMS2 1 MDI IETAKLEEH--LENQ---PSDPTNTYARPAEPVEEENKNGNGKPKSLSSG-LRKGTKKY  
 Hs SMSr 56 KRLMLSVRKLCQKIHIDVLEEMGYNSDSMGSMTPFISALQSTDWLCNGELSHDCDGPITQLNSDQ

TM1 D1-

Hs SMS1 114 EMIKIPMEPELERSQYPMEWGKTFLAFLYALSCFVLTVMISVVHERVPPKEVQPPLPDITFFDHF  
 Hs SMS2 56 PDYIGIAMPTESRNKFPHEWWKIGIAFIYAFNLVLTVMITVVHERVPPKELSPPLPKFFDYI  
 Hs SMSr 131 YQYMNIGKNKHSVRRLDP-EYWKTI LSCIVVFIVGFTSFIMVIVHERVPPDMQTYPPLPDITLDSV

TM2 D2-TM3

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 Hs SMS2 122 DRVKWAFSVSEINGTILVGLWITQWLFRLYKSI VGRRCFII GTLYLYRCITMYVTTLVPVGMHF  
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D3 TM4

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 Hs SMS2 187 QCAPKLNIGDSQAKVQRI RLISGGGSIITGSHILEGGDLFSGHTVTLTLTYLFIKEYSPRHFWY  
 Hs SMSr 260 OCTGKIYGSVWEKLHRAFAIWSGFEMTLTGVI-TCGDYMFSGHTVVTMTLNFFVTEYTPRSWNFL

TM5 D4-TM6

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 Hs SMS2 252 HLICWLLSAAGIICILVAHEHYTIDVITAYVTRIFFWYHSMANEKNLKVSSQTNFLSRAWWFP  
 Hs SMSr 324 HTLSWVLNLFGLFFFLAAHEHYSIDVITAYVTRIFFLYVHTLANTR---AYQSSRRARIV-FP

Hs SMS1 373 PFQYFEKNVQGI VPRSYHWPFPWP-VVHLISRQVKYSRLVNDT  
 Hs SMS2 317 IRYFFEKNVQGSIPCCESWPLSWPFCFKSSCKKYSRVQKIGEDNEKST  
 Hs SMSr 384 MRSFEFCNMGITVANEYCWPESKRAIKRIG

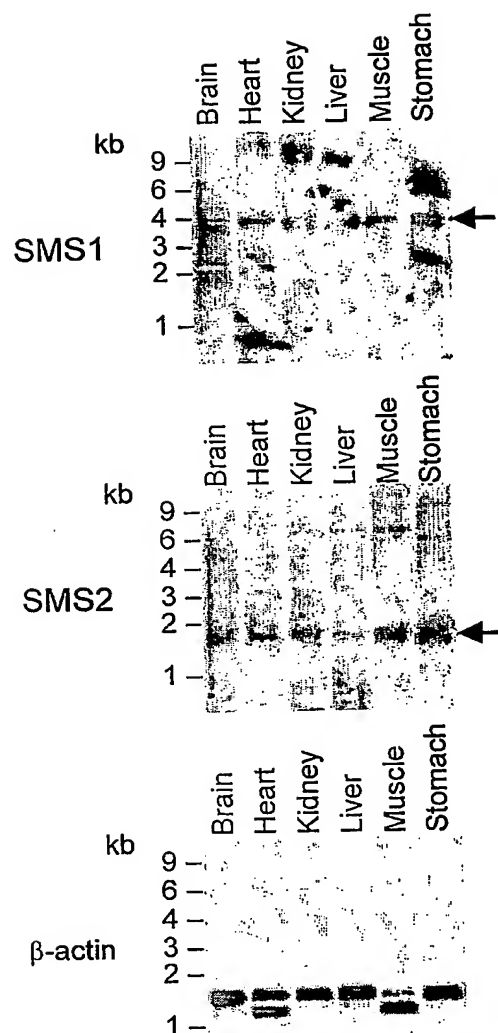
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	-D1-	D2	D3	D4
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Hs SMS2	112 PLPD	157 RRFCILGTLYLYRCITMYVT	221 CGDFLFSGHT	272 HYTDVITLAYYITRIFWWYH
Ce SMS1	175 PLPD	220 RRLCFILGSIILYGMRCITMYVT	288 CGDYIYSGHT	339 HYTDVILSYFACTRVFVAYH
Ce SMS2	88 PLPD	133 RRTFLGLAI MYGLRAVILGVT	202 CGDLMFSGHT	253 HYTMDVILAYWLSHVVFVSYH
Ce SMS3	65 PLPD	110 KRNVFCAGTLYAMRSVTLAAT	175 CGDILFSGHT	226 HYTDVILAYWLSNMVFRMYH
Pf SMS1	161 PLPD	211 IRIILLSFIYICIRSEFIYVT	266 CTDLVLSGHT	315 HYTDVILMGVFGGSVFLFYH
Pf SMS2	112 PLPD	161 CRFLYIVGSEYIRGFLIYVT	216 CADLIYSGHT	265 HYTSDVILGLFGVFMFSFYH
Hs SMSr	185 PLPD	230 RRLCSLMGTVELLRCTMEVT	293 CGDYMFSGHT	344 HYSIDVFIAYYITRIFFLYYH
Dm SMSr	232 PLPD	277 RRFALAGTVLLRCVIMLIT	350 CGDYMFSGHT	401 HYSIDVFIAYYITRIFFLYYH
Ce SMSr	214 PLPD	259 RRMFSLGTVLLRCVIMLIT	322 CGDYMFSGHT	373 HYSIDVFIAYYITRIFFLYYH
Ce SMSdr	111 PLPD	183 RRVFFCLAMAYSFRALCVTIF	247 CGDLIYSGHT	298 HYMDIVLCVTVSTRIFMEYH
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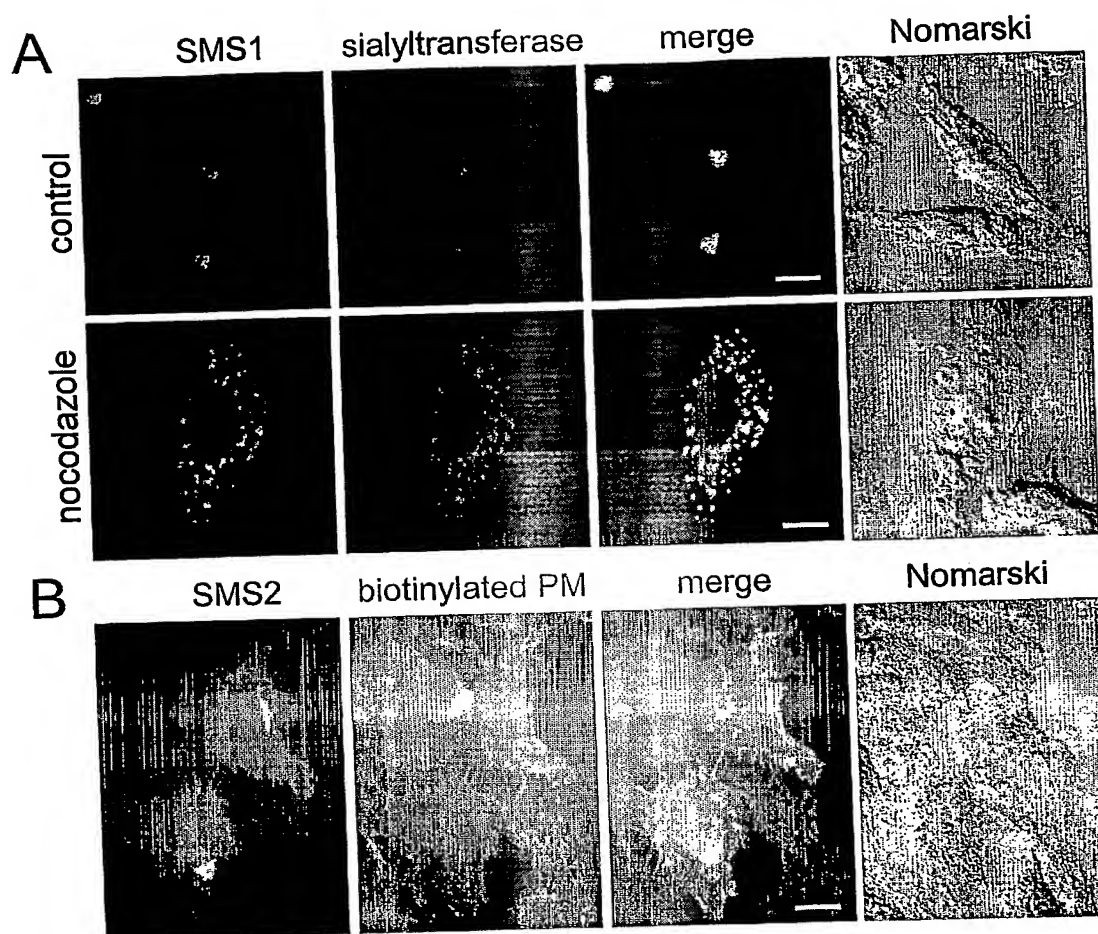
9/13

Fig. 10



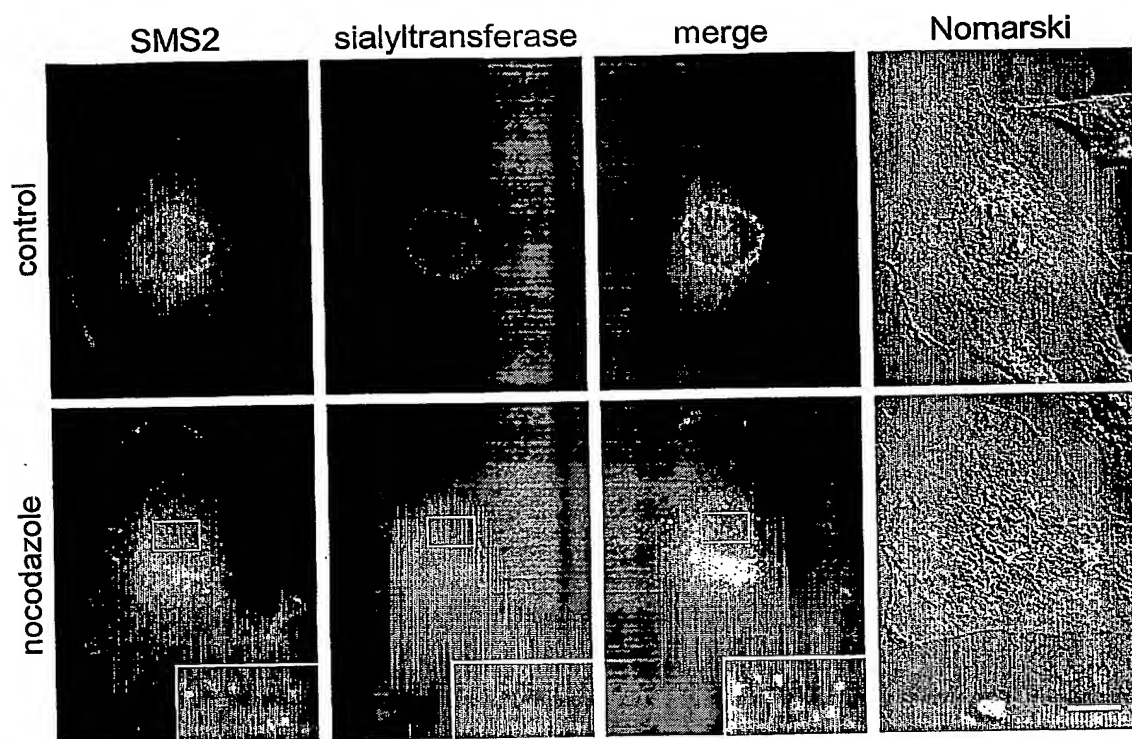
10/13

Fig. 11



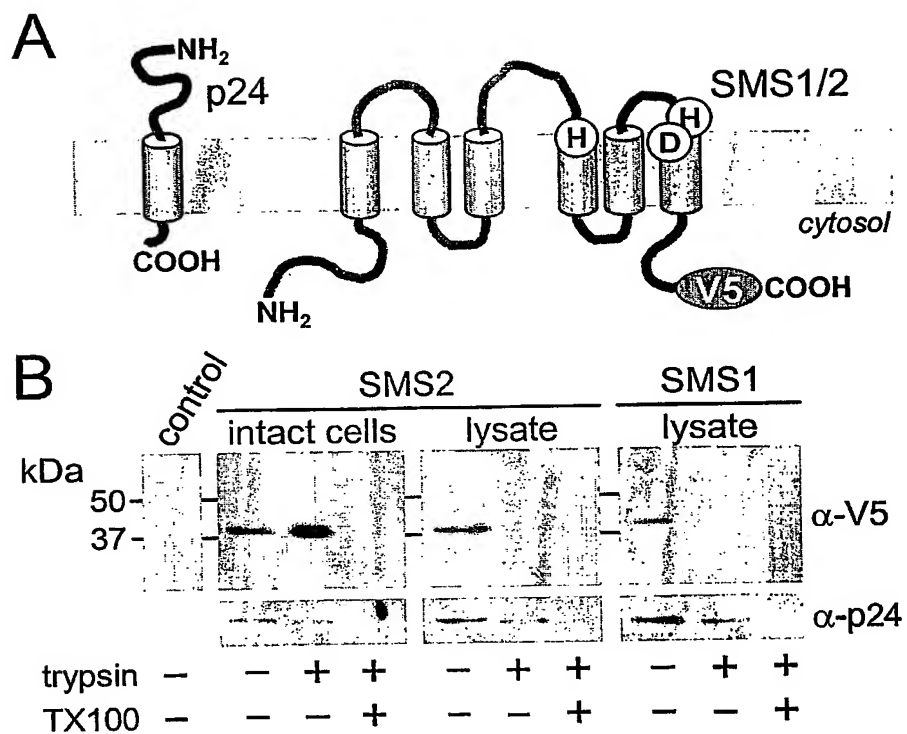
11/13

Fig. 12



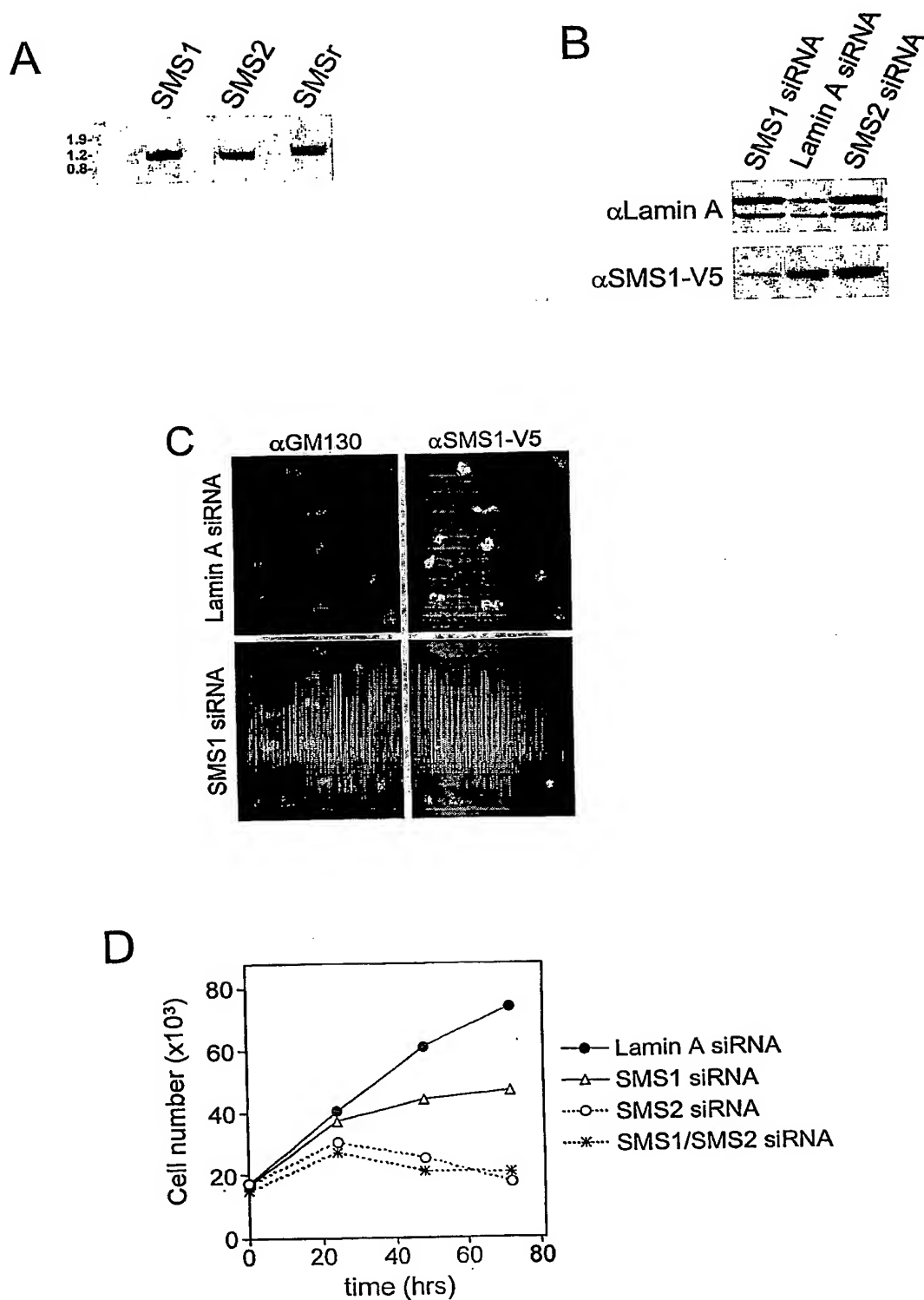
12/13

Fig. 13



13/13

Fig. 14



THE MOUNTAIN (1970)

# INTERNATIONAL SEARCH REPORT

International Application No  
PC1/NL2004/000488

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
IPC 7	C12N9/12	C12Q1/48 C12N15/54 C12N5/10 C12P7/64
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols)		
IPC 7 C12N C12Q C12P		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
EPO-Internal, Sequence Search, BIOSIS, WPI Data, PAJ, CHEM ABS Data, EMBASE, MEDLINE		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
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<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
25 April 2005		08.08.2005
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer  Bassias, I

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCI/NL2004/000488

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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X	<p>DATABASE Geneseq 'Online! 10 February 2003 (2003-02-10), "Human secretory polypeptide SPTM SEQ ID NO 835." XP002325876 retrieved from EBI accession no. GSN:ABP75651 Database accession no. ABP75651 see sequence -&amp; WO 02/083876 A (INCYTE GENOMICS, INC; DAFFO, ABEL; JONES, ANISSA, L; TRAN, ALANNA-PHUN) 24 October 2002 (2002-10-24) see in particular parts referring to human secretory polypeptide SPTM, SEQ ID NO: 835, claim 27</p>	1,2,4, 47,48, 54,55
X	<p>DATABASE Geneseq 'Online! 18 December 2003 (2003-12-18), "REMAP protein #18." XP002325877 retrieved from EBI accession no. GSN:ADC42858 Database accession no. ADC42858 see sequence -&amp; WO 03/027228 A (INCYTE GENOMICS, INC; LAL, PREETI, G; HONCHELL, CYNTHIA, D) 3 April 2003 (2003-04-03) see in particular parts referring to REMAP protein #18, claim 1, SEQ ID NO: 18</p>	1,2,4,7, 47,48
X	<p>DATABASE Geneseq 'Online! 1 October 2001 (2001-10-01), "Human bone marrow protein, SEQ ID NO: 346." XP002325878 retrieved from EBI accession no. GSN:AAM00870 Database accession no. AAM00870 see sequence</p> <p style="text-align: center;">-/--</p>	1,2,7, 47,48

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL2004/000488

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>&amp; WO 01/53453 A (HYSEQ, INC; FORD, JOHN, E; BOYLE, BRYAN, J; TANG, Y., TOM; LIU, CHENGH) 26 July 2001 (2001-07-26) see in particular parts referring to SEQ ID NO: 346 and claim 10</p> <p>-----</p> <p>DATABASE EPO Proteins 'Online! 2 February 2004 (2004-02-02), "Sequence 8703 from Patent WO0171042." XP002325879 retrieved from EBI accession no. EPOP:CQ580945 Database accession no. CQ580945 see sequence -&amp; WO 01/71042 A (PE CORPORATION) 27 September 2001 (2001-09-27) see in particular parts referring to sequence 8703</p>	<p>1,2,7, 47,48</p>
P,X	<p>HUITEMA KLAZIEN ET AL: "Identification of a family of animal sphingomyelin synthases." EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL, vol. 23, no. 1, 14 January 2004 (2004-01-14), pages 33-44, XP002325870 ISSN: 0261-4189 the whole document</p>	<p>1-35, 38-63</p>
P,X	<p>WO 03/073826 A (SAGRES DISCOVERY; MORRIS, DAVID, W) 12 September 2003 (2003-09-12) see the whole document, in particular claim 5, parts referring to mCP2702 and SEQ ID NO: 291 -&amp; DATABASE Geneseq 'Online! 18 November 2004 (2004-11-18), "Mouse protein sequence mCP2702." XP002325880 retrieved from EBI accession no. GSN:ABM85292 Database accession no. ABM85292 see sequence</p>	<p>1,2,4, 47,48</p>
P,X	<p>DATABASE Geneseq 'Online! 15 January 2004 (2004-01-15), "Human NOV13a SEQ ID 54." XP002325881 retrieved from EBI accession no. GSN:ADD49081 Database accession no. ADD49081 see sequence</p> <p>-/--</p>	<p>1,2,4, 47,48</p>

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## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/NL2004/000488

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	-& WO 03/060149 A (CURAGEN CORPORATION; GROSSE, WILLIAM, M; ALSOBROOK, II, JOHN, P; ANDER) 24 July 2003 (2003-07-24) see parts relating to NOV13a, SEQ ID NO: 54; claim 1; page 167 -----	1,2,4, 47,48
P,X	DATABASE Geneseq 'Online! 20 May 2004 (2004-05-20), "Human disease detection and treatment (MDDT) protein - SEQ ID 116." XP002325882 retrieved from EBI accession no. GSN:ADL22667 Database accession no. ADL22667 see sequence	1,2,4,7, 47,48
P,X	-& WO 03/062379 A (INCYTE GENOMICS, INC; JONES, ANISSA, L; DAHL, CHRISTOPHER, R; GIETZEN,) 31 July 2003 (2003-07-31) see in particular parts referring to MDDT protein, SEQ ID NO: 116, claim 27 -----	1,2,4,7, 47,48
P,X	YAMAOKA SHOHEI ET AL: "Expression cloning of a human cDNA restoring sphingomyelin synthesis and cell growth in sphingomyelin synthase-defective lymphoid cells" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 279, no. 18, 30 April 2004 (2004-04-30), pages 18688-18693, XP002325871 ISSN: 0021-9258 the whole document -----	1,2,4, 9-33, 38-63
P,A	LUBERTO CHIARA ET AL: "Purification, characterization, and identification of a sphingomyelin synthase from Pseudomonas aeruginosa. Plch is a multifunctional enzyme." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 278, no. 35, 29 August 2003 (2003-08-29), pages 32733-32743, XP002325872 ISSN: 0021-9258 -----	
A	LUBERTO CHIARA ET AL: "Sphingomyelin synthase, a potential regulator of intracellular levels of ceramide and diacylglycerol during SV40 transformation: Does sphingomyelin synthase account for the putative phosphatidylcholine-specific phospholipase" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 23, 5 June 1998 (1998-06-05), pages 14550-14559, XP002325873 ISSN: 0021-9258 -----	

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# INTERNATIONAL SEARCH REPORT

national application No.  
PCT/NL2004/000488

## Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 3, 5, 6, 8  
because they relate to subject matter not required to be searched by this Authority, namely:  
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☒ Claims Nos.: 3, 5, 6, 8  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-35, 38-63

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-35, 38-63

Polypeptides having the requested identity to the motifs as given in claim 1 or to the specific amino acid sequences as given in SEQ ID NOs: 12-22, with the mentioned enzymatic activity as described in claims 9-11, the corresponding nucleic acid sequences encoding said polypeptides, plasmids and microorganisms comprising said nucleic acid sequences processes and uses of the enzymes in question and the thereof produced products, methods for improving the yield of an secretion product in a cell and methods for targeting polypeptides in cellular compartments.

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2. claims: 36, 37

Process to isolate candidates for functional genes of a previously unidentified enzyme with known activity.

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## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Claims Nos.: 3,5,6,8

Claim 1 relates to polypeptides consisting of a sequence which has at least 80% identity to motifs which are defined mainly by variable amino acid residues. Motif (a) for example consists of 7 defined amino acid residues and 53-93 undefined amino acid residues. The requested percentage of identity is not limited to the defined part of the sequence but refers to the complete motif embracing also the undefined part of the motif. Any polypeptide falls into the scope of such a claim, since any amino acid sequence has 80% identity with the undefined part of the motif (and the resulting 20% non-identity with the fixed part of the motif). Consequently, no meaningful search could be performed for such an unclear claim and thus the search for claim 1 was limited to polypeptides having 100% identity to any of the motifs (a) ? (c). Furthermore, it has to be noted that claim 1 comprises numbers within the motifs which are nowhere explained and thus render the true scope of the claim unclear (Article 6 PCT). From the specific sequences 12-22 it could be derived that ?X(35,75)? must mean 35 to 75 variable amino acid residues (?X(8)? means 8 variable amino acid residues). Furthermore, the amino acids given in square brackets '!' where interpreted as possible alternatives at this position of the motif. Hence, claim 1 was searched with the above mentioned limitation and the interpretation of the unclear terms as given above.

Claim 3 refers to polypeptides having at least 70% similarity to sequences (SEQ ID NO: 1-11) with about 80% undefined amino acid residues. The requested similarity is not limited to the defined parts of the sequence but refers to the complete sequences, including the undefined sections. Hence any polypeptide having any sequence would fall into the scope of such a claim (see also argumentation given for the limited search of claim 1). Since no clear technical features existed for said claim, no meaningful search was possible.

Claim 5 referring to a polypeptide comprising an amino acid sequence with at least 20% identity to any of the SEQ ID NOs: 12-22 is so broad and thus unclear in the sense of Article 6 PCT. Polypeptides having an identity of only 20% with other polypeptides are not sufficiently supported in the description. Furthermore, it appears not very credible that polypeptides being only 20% identical to each other have the same biological function. If they have different functions, then they solve different technical problems. Due to these discrepancies, no meaningful search could be performed for claim 5. The same applies for claim 6 and claim 8, since identities of 22% or 30% are also not considered significant.

Continuation of Box II.2

Claims Nos.: 3, 5, 6, 8

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## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claim 1 relates to polypeptides consisting of a sequence which has at least 80% identity to motifs which are defined mainly by variable amino acid residues. Motif (a) for example consists of 7 defined amino acid residues and 53-93 undefined amino acid residues. The requested percentage of identity is not limited to the defined part of the sequence but refers to the complete motif embracing also the undefined part of the motif. Any polypeptide falls into the scope of such a claim, since any amino acid sequence has 80% identity with the undefined part of the motif (and the resulting 20% "non-identity" with the fixed part of the motif). Consequently, no meaningful search could be performed for such an unclear claim and thus the search for claim 1 was limited to polypeptides having 100% identity to any of the motifs (a) to (c). Furthermore, it has to be noted that claim 1 comprises numbers within the motifs which are nowhere explained and which render the true scope of the claim unclear (Article 6 PCT). From the specific sequences 12-22 it could be derived that "X(35,75)" must mean 35 to 75 variable amino acid residues ("X(8)" means 8 variable amino acid residues). Furthermore, the amino acids given in square brackets '!' where interpreted as possible alternatives at this position of the motif. Hence, claim 1 was searched with the above mentioned limitation and the interpretation of the unclear terms as given above.

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The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase

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**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

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**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

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